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Title	Protein-protein interactions of the DNA polymerase ð complex in the fission yeast Schizosaccharomyces pombe
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Qualification	PhD
Year	2002

Digitisation Notes:

- Page 130 appears twice in original

Protein-protein interactions of the DNA polymerase δ complex in the fission yeast *Schizosaccharomyces pombe*

by Javier Sanchez Garcia

Thesis presented for the degree of Doctor of Philosophy

at the University of Edinburgh

September 2002

A mis padres, por tantas razones.

"To think, I did all that, and may I say - not in a shy way, no, oh no
not me, I did it my way"

sung by Frank Sinatra

Declaration of Authenticity

I declare that this thesis was composed by myself and that the research presented is my own. Due acknowledgement is made within the text for the assistance of others.

Javier Sanchez
September 2002

Acknowledgements

I would like to thank my supervisor Stuart MacNeill for supervision, helpful discussions throughout this project, and critical reading of this manuscript.

I would like to thank all the people in the MacNeill and Adachi labs past and present for helpful discussions, help, support, critical manuscript reading, and helping me realise and remember that it was not just me (they know what I mean). Not enough room to mention them all but I would specially like to mention Ina Martin, Jess Worthington, Fiona Gray, Leo Ciufo, Do-Hyung Kim, Hiroyuki Tanaka, Ji-Young Kim, Alison Engles, Lindsay Anderson, Alan McNee and Fiona Pryde. As I said, there is no room to mention them all, so I hope no-one gets too offended. I would also like to thank all the members of the Ohkura, Brown, Hardwick and Beggs labs for helping me along the way.

Thanks to the BBSRC for their financial support.
I would also like to thank my second supervisor Peter Fantes for his help and support.

Special thanks are due to my parents for their support in many ways throughout this Ph.D., without them I would have been unable to get to this stage.

Thanks are also due to Rosie Mallin, for lots of reasons that she is aware of.

Abstract

In eukaryotes there are three essential DNA polymerases that are involved in the bulk of DNA replication: polα, polδ and polε. Polα is involved in generating a short RNA-DNA primer. Polδ and ε are involved in the elongation process of DNA replication. It has been suggested that polδ is the key enzyme that performs all of the processive DNA replication since the catalytic domains of polε are not essential.

In *S. pombe* polδ is comprised of four subunits: Pol3- the catalytic or A subunit, Cdc1- the B subunit, Cdc27- the C subunit, and Cdm1- the D subunit. Polδ in *S. cerevisiae* and mammals have homologues of these subunits, except for the D subunit of which there is no homologue in *S. cerevisiae*.

In this thesis polδ from *S. pombe* has been studied in two ways. One approach was to investigate the protein-protein interactions within polδ, and the other was to investigate Cdc1, the highly conserved B subunit of unknown function. The protein-protein interactions were investigated using a combination of two-hybrid assays and mutational analysis. Cdc1 was investigated by performing extensive mutational analysis using both random and site directed methods.

The combination of approaches has demonstrated that the C- terminal ZnF2 region of both *S. pombe* and *S. cerevisiae* A subunit (Pol3) is involved in the direct binding to the B subunit. The four cysteines present in the zinc finger are involved in maintaining the structure of both *S. pombe* and *S. cerevisiae* ZnF2. Mutational analysis of Cdc1 (the *S. pombe* B subunit) has identified a conserved region (DomIII) that could be involved in the function of Cdc1. Additionally, binding assays with the Cdc1 mutants have suggested a region of Cdc1 (from amino acids 293 to 329) as being involved in the binding to Pol3.

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Abbreviations

~	approximately
°C	degree Celsius
µ-	micro
µF	micro Farad
3-AT	3-amino 1,2,4 Triazole
A	adenine
aa	amino acids
ACS	ARS consensus sequence
ade	adenine
AMPS	ammoniumperoxodisulphate
ARS	autonomously replicating sequence
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
C	cytosine
cdc	cell division cycle
CDK	cyclin-dependent protein kinase
dH_2O	water (distilled)
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
EDTA	ethylen-diamine-tetraacetic acid
EMM	Edinburgh minimal medium
exo^-	exonuclease deficient
FRYL	Fromont-Racine yeast library
G	guanine
g	gram
GST	glutathione-S-transferase
hys	hydroxyurea sensitive
kb	kilobase
kDa	kilodalton
kV	kilovolt
l	litre
LB	Luria broth
leu	leucine
LiAc	lithium acetate
M	molar
m-	milli
max.	maximum
Mb	megabase
MCM	mini chromosome maintenance
MCS	multiple cloning site
ME	malt extract
mis	minichromosome instability
mRNA	messenger RNA
OD	optical density

ONPG	*o*-nitrophenyl-beta-D-galactopyranoside
ORC	Origin Recognition Complex
ORF	open reading frame
ori	origin of replication
PAGE	polyacrylamide gel electrophoresis
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PEG	polyethyleneglycol
pol	DNA polymerase
pre-RC	pre- replication complex
PVDF	polyvinylidene difluoride
res	resistant
RFA	replication factor A
RF-C	replication factor C
RIP	replication initiation point
RNA	ribonucleic acid
RPA	replication protein A
rpm	revolutions per minute
SAP	shrimp alkaline phosphatase
SDS	sodium dodecylsulphate
ssDNA	single stranded DNA
T	thymine
t.s.	temperature-sensitive
TCA	trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine
tet	tetracycline
U	uracil
ura	uracil
UV	ultraviolet light
v/v	volume per volume
w.t.	wild type
w/v	weight per volume
YE	yeast extract
YMM	yeast minimal media
YPDA	yeast peptone dextrose adenine media
Ω	ohm (unit of resistance)

1 Introduction

1.1 DNA Replication and the Cell Cycle

An integral part of the cell cycle is DNA replication, a complex process involving the co-ordinated action of many enzymes, structural proteins and associated factors. The end result is the production of a complete and accurate copy of the entire chromosomal DNA and it is regulated so that it occurs only once per cell cycle. The cell cycle can be divided into 4 phases: G1, S, G2 and M. During the S or synthesis phase DNA is replicated, whilst mitosis occurs during the M phase; G1 and G2 (or Gap1 and Gap2) are gaps between the S and M phases. For cells to enter S phase and replicate their DNA they need to pass a point in G1 termed Start after which the cell is committed to replicate its DNA. This commitment requires many factors such as Cdc2 activity and transcription of various genes including *cdc18*. After the cell has progressed through Start the initiation of DNA replication begins.

DNA replication can be divided into three stages: Initiation, Elongation, and Maturation, each of which will be discussed in turn.

1.2 Initiation

Eukaryotic DNA replication begins at many sites, called origins, along each chromosome, allowing replication to be completed more rapidly. Each origin forms a focus at which all of the factors associated with DNA replication can localise. Origins are tightly controlled so that they fire once and only once every cell cycle.

Work on budding yeast identified chromosomal sequences which, when placed in plasmid DNA, conferred on it the ability to replicate (Stinchcomb *et al.,* 1979). These sequences were, as a result, termed Autonomously Replicating Sequences (ARS). This work however, could not conclude if these ARS were in actual fact origins of replication in chromosomes, or if they only behaved as origins in plasmid DNA. The development of a method involving the use of two dimensional gels and

Southern hybridisation (reviewed in Brewer and Fangman, 1991) provided a great deal of information in this respect. This method allowed the localisation of origins to particular sites in the chromosome. Further, if the origin is not in the site investigated the method can provide information as to whether the origin is nearby. There have been sequences identified as ARS that were not found to be origins in chromosomes (Brewer and Fangman, 1991). The most common of these are due to chromosomal location interference, when the ARS is located near the centromere or the telomere (Brewer and Fangman, 1991).

Investigation of the origin sequence in budding yeast has identified several components of the origins. Origins can be divided into four regions: A, B1, B2, and B3 (reviewed in Kelly and Brown, 2000). Region A is the ARS consensus sequence (ACS), which is highly conserved and is also required for the function of the origin. The B elements are less conserved and are found adjacent to region A. Region A is a binding site for the Origin Recognition Complex (ORC, discussed below) and it is thought that ORC also binds to region B1. These regions of the budding yeast origin are localised in 100-200 bp (Kelly and Brown, 2000).

In *S. pombe* the origins were found to be more complex than in *S. cerevisiae* (Kelly and Brown, 2000). They were found to be much larger (500-1000 kb in *S. pombe* as opposed to 100-200 bp in *S. cerevisiae*), and sequence analysis found little homology between origins except for the presence of sequence clusters 20-50 bp in length that were AT rich (Kelly and Brown, 2000). The numbers of these clusters were found to vary between origins. However, no regions resembling those in the origins of *S. cerevisiae* (see above) were found in *S. pombe* (Kelly and Brown, 2000). In higher eukaryotes fewer origins have been characterised and those that have, have been to low resolution only. In *Xenopus* and *Drosophila* egg extracts, origins appear to have little sequence specificity and in the human β- globin gene it appears that several kilobases contribute to the origin (Kelly and Brown, 2000).

The development of Replication Initiation Point (RIP) mapping (Bielinsky and Gerbi, 1998) allowed the mapping of origins to the base pair from which the

replication fork starts i.e. it enabled identification of where in the actual origin the replication fork starts. In the *S. cerevisiae* ARS1 origin it was found that it starts at a point between the B1 and B2 regions, very close to ORC binding. In the *S. pombe* ars1 origin and in the human lamin B2 origin it was found that even though the origin is very large replication starts at a single point (Bielinsky and Gerbi, 2001a).

A protein complex, termed the Origin Recognition Complex (ORC), that was able to bind to the A and B1 regions of the origin of *S. cerevisiae*, was identified and, as a result of this binding, was thought to be involved in initiation. Further, it appeared that the ORC was present in most eukaryotes as ORC subunits or homologous sequences were found in other organisms such as *S. pombe*, humans, *Drosophila* and *Xenopus* (Kelly and Brown, 2000). The importance of ORC in initiation was shown when mutants in ORC subunits of *S. pombe*, *S. cerevisiae* or *D. melanogaster* were defective in initiation (Kelly and Brown, 2000). Further, in *Xenopus*, immunodepletion of Orc1 and Orc2 results in an inability of the *Xenopus* extract to enter DNA replication (Carpenter *et al.,* 1996; Rowles *et al.,* 1996).

In *S. cerevisiae* ORC is a six protein complex, Orc1 to Orc6 (Kelly and Brown, 2000). Four of the Orc subunits are involved in the direct binding to DNA (Orc1, 2, 4 and 5; (Bell, 2002)). Orc3 appears not to be involved in the direct binding to DNA although it is required for binding, and Orc6 is not required for binding although it is required *in vivo* (Bell, 2002). In *S. pombe*, ORC is also formed of six subunits (SpOrc1-6; (Moon *et al.,* 1999)), but, unlike the other ORCs identified, one of the subunits, SpOrc4, has a DNA binding domain (Bell, 2002). This domain binds AT-rich DNA and is called an AT- hook. Due to the fact that origins in *S. pombe* have no identified consensus sequence, it has not been possible to define a region of the origin that is required for ORC binding in the same way that the A region of *S. cerevisiae* origins has been shown to be required for ORC binding.

As mentioned above, ORC functions in initiation, as discovered by mutational and immunodepletion studies (Kelly and Brown, 2000; Carpenter *et al.,* 1996). However, ORC is bound to the ARS throughout the cell cycle, hence, there must be additional

factors influencing the initiation of replication (Diffley *et al.,* 1994). It is believed that the function of ORC is that of a recruitment factor, that recruits these additional factors to the origin. ORC has been found to interact directly with some of these factors, Cdc6 being one of them (see below) whose interaction with ORC has been extensively studied (Bell, 2002). Also ORC is thought to have roles other than those at initiation, such as roles in M phase, chromosome condensation and in heterochromatin establishment (Bell, 2002).

Once the origin has been identified and ORC has bound to it, the DNA must be unwound. Since ORC probably acts as a recruitment factor (see above) it is most likely that it will recruit a helicase to unwind the origin. In the SV40 viral DNA replication system, a model system for studying DNA replication in higher eukaryotes, unwinding of the origin is brought about by the binding of T antigen to the origin in an ATP-dependent manner (Borowiec *et al.,* 1990). In yeast there are several lines of evidence to suggest that the unwinding of the origin, and subsequent unwinding of the replication fork, is carried out by Minichromosome Maintenance proteins (MCM proteins). The MCM proteins are a group of proteins which are required for minichromosome replication and of these, at least six are required for eukaryotic DNA replication (MCM2 to MCM7). These proteins form a hexameric complex. In humans intermediate complexes of MCM4, MCM6 and MCM7 with loosely associated MCM2 have been found (Ishimi, 1997). In both *S. cerevisiae* and *S. pombe* MCM proteins are associated with replication origins and are thought to move along with the replication fork (Leatherwood, 1998; Aparicio *et al.,* 1997; Ogawa *et al.,* 1999). Furthermore, in HeLa cells a complex of MCM4, MCM6 and MCM7 has been found to have helicase activity (Ishimi, 1997). Interestingly, only complexes lacking MCM2 were found to have helicase activity, so it appears that MCM2 inhibits the helicase function. MCM2 is however, only loosely associated with the complex, hence, it can dissociate easily from it (Ishimi, 1997) which could act as a regulatory mechanism. Further, MCM proteins, as expected for helicases, have an ATPase motif present in each of the six subunits (Leatherwood, 1998). It seems plausible therefore, that the MCM proteins are indeed the helicases that unwind the origin and the replication fork.

MCM proteins form a hexamer that is ring shaped, so they require to be loaded onto the DNA (Leatherwood, 1998). In the case of the MCM complex from HeLa cells described above (MCM4, MCM6 and MCM7) it is thought that these three proteins form a trimeric complex that interacts with another one to form a hexamer (Ishimi, 1997). The loading of the MCM onto the DNA involves the MCM loader (Cdc6 in *S. cerevisiae*, Cdc18 in *S. pombe*; (Leatherwood, 1998)). It was found that the MCM proteins need both ORC and the MCM loader to be associated with the origin DNA. On the other hand, the MCM loader needed only ORC and not the MCM proteins to be associated with the origin DNA (Kelly and Brown, 2000). Hence, it is thought that the MCM loader binds to the ORC after it is bound to the origin and then it loads the MCM proteins onto the DNA. After the MCM proteins are loaded, dissociation of the MCM loader does not affect their localisation. If ORC were to dissociate it does not affect the localisation of MCM proteins either (Kelly and Brown, 2000). Recently it was found that Cdt1 is also involved in the localisation of the MCM proteins (Lei and Tye, 2001). Cdt1 was found to be able to interact directly with Cdc18 (the MCM loader in *S. pombe*) and it is needed for the localisation of the MCM proteins as is ORC and Cdc18. Also as is the case for ORC and Cdc18, the dissociation of Cdt1 after loading of the MCM proteins does not affect their localisation (Lei and Tye, 2001). Cdt1 appears to be present in other eukaryotes: DUP in *Drosophila* (Lei and Tye, 2001), Tah11/Sid2 in *S. cerevisiae* (Devault *et al.*, 2002) and it was also identified as RLF-B in *X. laevis* (Tada *et al.*, 2001).

The binding of ORC, MCM proteins and MCM loader to the origin of replication results in the formation of a pre-replication complex (pre-RC), as in Figure 1.1 below.

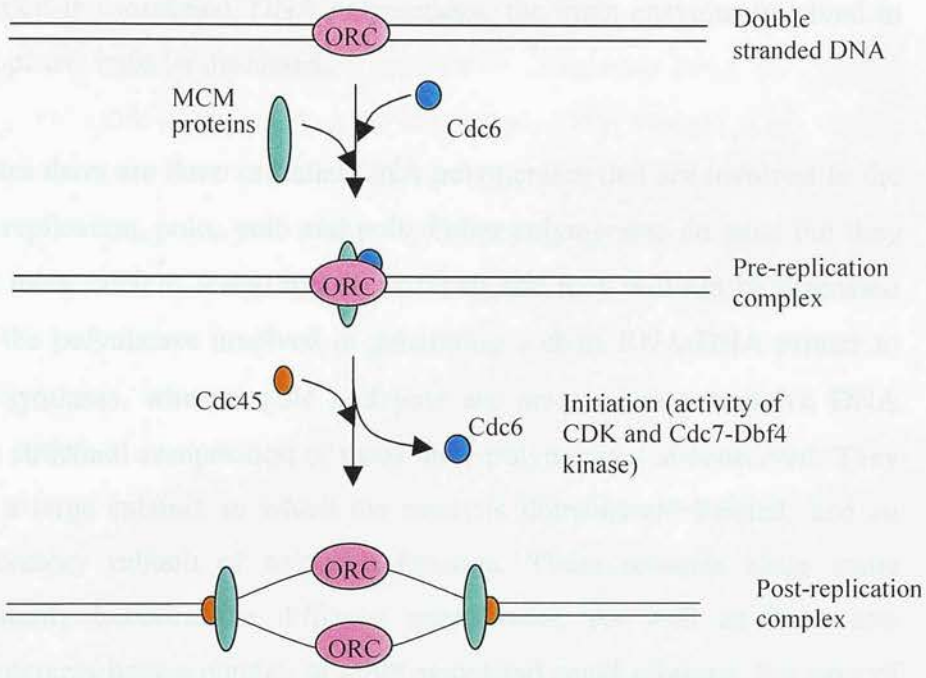

Figure 1.1. Summary of the assembly and activation of the pre-RC to produce the post-replication complex. Adapted from Bielinsky and Gerbi (2001a). See text for further details.

After the pre-RC is formed, activation is brought about by a number of factors, amongst which are the activity of protein kinases CDK and Cdc7-Dbf4 kinase (Kelly and Brown, 2000). The activation of the pre-RC also causes the binding of Cdc45 to the MCM proteins. Cdc45 is thought to be able to move along the fork and it has been shown to be able to bind to both MCM proteins and polα simultaneously ((Kelly and Brown, 2000); see section 1.3.1, for a discussion of polα). This is thought to tether the MCM proteins to polα .

After the origin has been recognised, helicases are loaded that unwind the origin, Cdc45 is also loaded, it directs the association of polα with the helicases. Now the actual replication of the DNA can begin.

1.3 DNA polymerases

Before elongation is considered, DNA polymerases, the main enzymes involved in the elongation phase, must be discussed.

In all eukaryotes there are three essential DNA polymerases that are involved in the bulk of DNA replication, polα, polδ and polε. Other polymerases do exist but they have different roles, such as lesion bypass or repair and they will not be discussed here. Polα is the polymerase involved in generating a short RNA-DNA primer to initiate DNA synthesis, whereas polδ and polε are involved in processive DNA synthesis. The structural composition of these three polymerases is conserved. They all consist of a large subunit, in which the catalytic domains are located, and an associated accessory subunit of unknown function. These subunits share some sequence similarity between the different polymerases. As well as these two subunits, polymerases have a number of other associated small subunits. For ease of reference, the subunits of the polymerases in this thesis will be referred to as A subunits (for the catalytic subunit), B subunits (the accessory subunit) and so on (this nomenclature is based upon that proposed by MacNeill *et al.*, (2001b)). Table 1.1 below shows examples of the subunit composition of polα, δ and ε taken from a number of organisms.

		A (Catalytic)	B	C	D
Polα	*(Mammalian*[a]*)*	Pol1 (180)	Pol2 (86)	Pri1 (58)	Pri2 (48)
	(Cerevisiae)[b]	Pol1 (167)	Pol12 (79)	Pri2 (62)	Pri1 (48)
	(Pombe[c]*)*	Pol1	Spb1	Spp1	Spp2
Polδ	*(Mammalian*[d]*)*	PolD1 (125)	PolD2 (48)	p66	p12
	(Cerevisiae[e]*)*	Pol3 (125)	Pol31 (58)	Pol32 (55)	-
	(Pombe[f]*)*	Pol3 (125)	Cdc1 (55)	Cdc27 (54)	Cdm1 (22)
Polε	(Human[g])	p261	p59	p12	p17
	(Cerevisiae[h]*)*	Pol2 (256)	Dpd2 (80)	Dpb3 (34)	Dpb4 (29)
	(Pombe[i]*)*	Pol2	Dpb2 (67)[j]		Dpb4 (24)[k]

Table 1.1. Structures of eukaryotic DNA polymerases. Numbers in brackets indicate sizes in kDa, when name of subunit is pXX, XX denotes the size in kDa. Note the conserved structure of a large catalytic subunit and small accessory subunit. See text for further details. a) (Copeland and Wang, 1993) b) (Burgers, 1998) c) (MacNeill and Burgers, 2000) d) (Lee *et al.*, 1984; Liu *et al.*, 2000a; Zhang *et al.*, 1991; Chung *et al.*, 1991; Hughes *et al.*, 1999b), e) (Bauer *et al.*, 1988) f) (Zuo *et al.*, 1997; Zuo *et al.*, 2000c) g) (Kesti *et al.*, 1993; Li *et al.*, 2000) h) (Dua *et al.*, 2002) i) (D'Urso and Nurse, 1997) j) SPBP8B7.14c k) SPBC3D6.09. Note that strictly speaking Pri1 and Pri2 (or Spp1 and Spp2) of polα are not components of the DNA polymerase and hence not the C or D subunit.

1.3.1 DNA polymerase α

Polα is an essential enzyme required for DNA replication in the SV40 model system (Tsurimoto and Stillman, 1991). It has a moderately processive DNA polymerase activity and an associated primase activity (Fisher *et al.,* 1979; Plevani *et al.,* 1984). In contrast to polδ and ε (see below) polα does not have an associated 3' to 5' exonuclease activity (Kaguni *et al.,* 1984), which means that this enzyme is error prone as it cannot backtrack and correct its own errors. It is formed from four subunits; Pol1 (180 kDa), the A subunit, Pol12 (86 kDa) the B subunit, Pri1 (58 kDa) and Pri2 (48 kDa). The A subunit, Pol1, is very conserved (Miller *et al.,* 1988) and is the subunit in which the polymerase functions of the enzyme are located. As the polymerase is only moderately processive it will not replicate long stretches of DNA. The primase activity is located in the remaining two subunits (Pri1 and Pri2) which are not part of the DNA polymerase and hence not the C or D subunits. These associated polymerase/primase enzymatic activities allows polα to generate a short RNA primer on single stranded DNA of approximately 30 nucleotides in length (Fisher *et al.,* 1979; Nasheuer and Grosse, 1988). This is brought about by RF-C which causes polα to stop when the primer is about 30 nucleotides in length. (Mossi *et al.,* 2000). Polα is known to interact with Cdc45 (Uchiyama *et al.,* 2001). This interaction is thought to be responsible for the loading of polα onto the replication complex where it can generate the primer. It has recently been shown that the loading of polα is most likely a two step process. The first step is the loading of polα-Cdc45 onto a nuclear structure. Cdc45, via its interaction with the MCM proteins, then loads polα onto the replication complex (Uchiyama *et al.,* 2001).

1.3.2 DNA polymerase ε

Polε is involved in the replication of the bulk of DNA (D'Urso and Nurse, 1997; Sugino *et al.,* 1998; Pospiech *et al.,* 1999). It is formed from four subunits, the catalytic subunit, Pol2 (256 kDa); Dpb2 the B subunit (80 kDa); Dpb3 (29 kDa) and Dpb4 (29 kDa) in *S. cerevisiae* (Dua *et al.,* 2002). Pol2 has an intrinsic highly processive polymerase activity in the absence of Proliferating Cell Nuclear Antigen

(PCNA, see section 1.3.4.1; (Dua *et al.,* 2002)). Polε however, does interact with PCNA, most likely via a PCNA binding motif located in the catalytic subunit (Dua *et al.,* 2002), which stimulates the processivity of polε. Further, polε can be inhibited by high KCl concentrations but in the presence of PCNA, polε is not inhibited and can perform DNA synthesis. Polε also has a 3'→5' exonuclease activity, so it can backtrack and repair its own errors (Morrison and Sugino, 1994), which gives a high fidelity to this enzyme.

Polε has been shown to be essential, as the deletion of the catalytic subunit (Pol2) in *S. cerevisiae*, proved to be lethal (Morrison *et al.,* 1990). Also, in *S. pombe*, cells with a disruption of the catalytic subunit of polε proved unable to support growth (Sugino *et al.,* 1998). Even though polε is essential, its polymerase and 3'→5' exonuclease activities are not. Deletion of the catalytic domains (polymerase and exonuclease domains) in *S. cerevisiae* by Kesti *et al.* (1999a) showed that the cells were viable and even though the cell displayed retarded growth it was comparable to wild type with respect to DNA replication, DNA repair, and sensitivity to DNA damaging agents. This suggests that polε might have an essential function other than that of a polymerase or exonuclease. Deletion of the whole catalytic subunit makes cells unable to support growth, whereas the catalytic domain, located at the N-terminus, is not required for viability. This suggests that this other function may be located in the C-terminus of the catalytic subunit. Interestingly, the C- terminus contains two putative zinc finger motifs. In *S. pombe* similar work was carried out (Feng and D'Urso, 2001) but it was found that after the catalytic domain was deleted the cells were sensitive to DNA damaging agents and had an elongated S phase (as opposed to work in *S. cerevisiae* (Kesti *et al.,* 1999a) in which it was shown that the N- terminal deletion was not sensitive to DNA damaging agents; (Feng and D'Urso, 2001)), which suggests either a role for polε in repair or alternatively, that the deletion mutant is somehow responsible for introducing more mutations to the DNA. Furthermore, when the N- terminus (containing the catalytic domains) was expressed *in trans* it was discovered that the phenotype of the cells was indistinguishable from wild type. This suggests that the N- terminus of polε is able to carry out its function even without being covalently attached to the C- terminus. Interestingly however,

even though a deletion of the N- terminal domain was viable, point mutations made in the catalytic domain *S. cerevisiae* (Dua *et al.,* 1999) were not viable. Therefore, it is believed that the catalytic functions of polε are not essential, and when the catalytic domain is not present another polymerase is able to perform the same function as polε. However, if the catalytic functions are merely disrupted instead of deleted, polε will be localised as normal but will not carry out DNA replication, which is likely to result in the enzyme obstructing, and hence inhibiting, the other substitute polymerase.

In summary, it appears that polε has a function in replicating the bulk of the DNA but this function appears not to be essential. It is most likely that polε has another essential function which involves the C- terminus of the protein.

1.3.3 DNA polymerase δ

DNA polymerase δ has been identified and purified in mammals, (Lee *et al.,* 1984; Liu *et al.,* 2000a; Zhang *et al.,* 1991; Chung *et al.,* 1991; Hughes *et al.,* 1999b), in *S. cerevisiae* (Bauer *et al.,* 1988; Gerik *et al.,* 1998) and in *S. pombe* (Zuo *et al.,* 1997). It is an essential enzyme that has both a polymerase activity and a 3'→5' exonuclease activity, which means that this is a high fidelity enzyme able to backtrack and correct its own errors. On its own it is not processive, and will dissociate from the DNA after synthesising only a short stretch of DNA. It is processive only when it is associated with Proliferating Cell Nuclear Antigen (PCNA; see section 1.3.4.1), which helps differentiate this enzyme from polε which is processive even in the absence of PCNA. Experiments in which the catalytic domain of polε was shown to be non-essential (Kesti *et al.,* 1999a; Feng and D'Urso, 2001) have shown that polδ is apparently able to perform all of the processive DNA synthesis. In *S. cerevisiae* the transcription of components of polδ are induced at the start of the S phase, during which DNA replication occurs. This, however, is not the case in *S. pombe*, where polδ transcripts and protein levels appear to be constant

throughout the cell cycle (Park *et al.*, 1993). Therefore, it appears that expression of polδ is not regulated in the same way in both budding and fission yeast.

In *S. pombe* and mammals, pol δ is formed from four subunits; the catalytic or A subunit, which is where the polymerase and exonuclease domains are found, the B subunit of unknown function, and the C and D subunits. In *S. cerevisiae* polδ is formed from three subunits that are homologous to the A, B and C subunits, but a homologue of the D subunit (present in *S. pombe* and mammals) has not been found. There is significant homology between the subunits of polδ in the different organisms and it is highest in the A subunit. The D subunit (when present) has only a low degree of conservation and this is restricted to a region of the C- terminus.

1.3.3.1 *Schizosaccharomyces pombe* polδ

In *S. pombe* polδ is composed of four subunits Pol3, Cdc1, Cdc27 and Cdm1 (Zuo *et al.*, 1997; Zuo *et al.*, 2000c), see Figure 1.2 below.

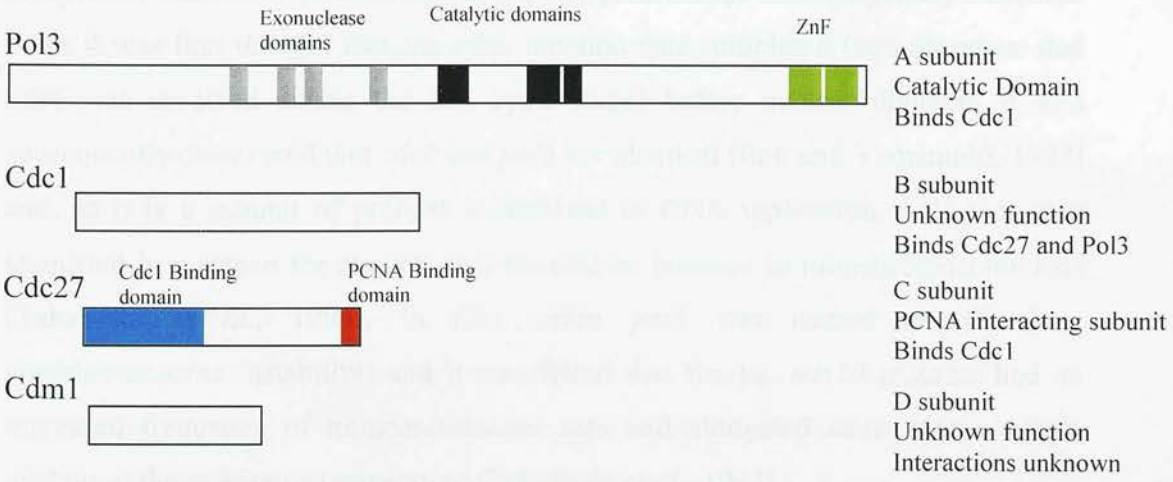

Figure 1.2. Subunit composition and interactions of *S. pombe* polδ. ZnF is a region formed from two putative zinc finger motifs. See text for further details.

1.3.3.1.1 Pol3

Pol3 is a 125 kDa protein encoded by the essential *pol3* gene where the catalytic function of polδ is located (Francesconi *et al.,* 1993). It has polymerase activity and 3'→5' exonuclease activity, which allows pol δ to correct its own errors as it replicates the DNA (Morrison and Sugino, 1994). Contained within its C-terminus are two putative zinc finger regions of unknown function.

The *pol3* gene was isolated by a number of groups in different screens. It was isolated by both Pignede *et al.* (1991) and Park *et al.*, (1993) by utilising the sequence of the *S. cerevisiae* homologue *pol3* to generate a probe used to screen *S. pombe* genomic libraries. It was also defined in a screen that attempted to identify cell division cycle (*cdc*) mutants (Nurse *et al.,* 1976) and in this screen *pol3* was named *cdc6*. The *cdc6* mutant was found to arrest at the restrictive temperature of 35°C with highly elongated cells, indicative of a defect in either DNA synthesis or nuclear division (described in Nurse *et al.,* 1976). As well as the elongated cells the *cdc6* mutant cells also showed a single nucleus and a 2C DNA content. The transition point, which defines the point in the cell cycle at which the *cdc* function is completed, was determined (Nurse *et al.,* 1976). From the relatively early transition point it was first thought that the *cdc6* function was completed (and therefore that *cdc6* was involved during the cell cycle stage) before nuclear division. It was subsequently discovered that *cdc6* and *pol3* are identical (Iino and Yamamoto, 1997) and, as it is a subunit of polδ, it is involved in DNA replication. *pol3* was also identified in a screen for mutants that showed an increase in minichromosome loss (Takahashi *et al.,* 1994). In this screen *pol3* was named *mis10*$^{+}$ (*mis*, *m*inichromosome *in*stability) and it was found that the t.s. *mis10* mutants had an increased frequency of minichromosome loss and elongated cells with a single nucleus at the restrictive temperature (Takahashi *et al.,* 1994).

The *S. cerevisiae* homologue of Pol3 was identified as a part of a protein complex that had polymerase activity but was different from polymerases α and ε. In this complex there was a protein that was thought to be of 140 kDa and was thought to be the catalytic subunit (Bauer *et al.,* 1988). It was then shown that the catalytic subunit

was in fact 125 kDa in size and that it was encoded by the *cdc2* gene (Boulet *et al.*, 1989; Sitney *et al.*, 1989). The *CDC2* gene in *S. cerevisiae* was identified in a screen similar to the *cdc* screen in *S. pombe* (described briefly above). It was found that the *CDC2* mutant *S. cerevisiae* cells were sensitive to hydroxyurea and arrested with a single bud with short spindles and a single nucleus located at the proximity of the neck that separates the mother and daughter cells (described in Pringle and Hartwell, 1981). Characterisation of cells with *CDC2* mutations demonstrated that these cells did not have polδ activity (Boulet *et al.*, 1989; Sitney *et al.*, 1989). Further, it was also demonstrated that *CDC2* mutants were unable to replicate their DNA efficiently (Budd and Campbell, 1993). Later it was proposed (and accepted) that the name *CDC2* be changed to *POL3* (Burgers *et al.*, 1990).

The mammalian A (or catalytic) subunit was identified as a protein of 125 kDa in size that was associated with another smaller protein and showed DNA polymerase δ activity (Lee *et al.*, 1984). The cDNA of the catalytic subunit of bovine polδ has been cloned (Zhang *et al.*, 1991) also the A subunit of human polδ has been cloned (Chung *et al.*, 1991) and they share 94% identity. There is a high degree of conservation between the A subunits. *S. cerevisiae* and *S. pombe* have 52% identity (Pignede *et al.*, 1991), *S. cerevisiae* and bovine have 44% identity (Chung *et al.*, 1991).

Temperature sensitive (t.s.) mutations in *pol3* in *S. pombe* were investigated (Francesconi *et al.*, 1993). Cell growth in these mutants arrested at the restrictive temperature and it was shown that the cells arrested with long cells at S phase. Mutations in the exonuclease domains of *S. cerevisiae pol3* have shown that these domains are important for exonuclease activity. If they are mutated cells do not have exonuclease activity and have increased rate of mutations (Simon *et al.*, 1991). Some t.s. mutations in the C- terminal zinc finger region of *S. cerevisiae pol3* are able to be rescued by a mutation of the B subunit (Giot *et al.*, 1995; Giot *et al.*, 1997). Interestingly, it was discovered that an overexpression of *S. pombe* Pol3 caused a retardation of growth indicating that increased levels of Pol3 have a detrimental effect on the cell (MacNeill *et al.*, 1996).

A study was done to investigate the ability of *S. pombe* and *S. cerevisiae* Pol3 proteins to complement across species (Moussy *et al.,* 1995). Results showed that *S. pombe* Pol3 protein was not able to rescue growth of a strain deleted for *S. cerevisiae pol3*. Further, *S. cerevisiae* Pol3 was not able to rescue t.s. mutations in *S. pombe pol3*. Chimeras, combining different regions of *S. pombe* and *S. cerevisiae* Pol3 were also tested for the ability to rescue *S. cerevisiae pol3* deletions or *S. pombe* t.s. mutations in *pol3*. Results seem to indicate that the C terminus of *S. pombe* Pol3 is necessary for the rescue of the *S. pombe* mutants and that the same is the case in *S. cerevisiae*: the C- terminus of *S. cerevisiae* Pol3 is needed for the rescue of *S. cerevisiae* mutants. This region is comprised of conserved boxes CT-1, CT-2, CT-3, but more interestingly also contains the two putative C- terminal zinc fingers, which could be an indication of the importance of these two zinc fingers.

1.3.3.1.2 Cdc1

Cdc1, the B subunit, is an essential protein 55 kDa (462 amino acids) in size (MacNeill *et al.,* 1996) which was first defined in a screen that attempted to identify *cdc* mutants (Nurse *et al.,* 1976). The *cdc1* mutant was found to arrest at the restrictive temperature of 35°C with highly elongated cells, indicative of a defect in either DNA synthesis or nuclear division (described in Nurse *et al.,* 1976). As well as the elongated cells the *cdc1* mutant cells also showed a single nucleus and a 2C DNA content. From the transition point it was originally thought that *cdc1* was involved in nuclear division. *cdc1* was also identified in a screen for mutants that showed an increase in minichromosome loss (Takahashi *et al.,* 1994). In this screen *cdc1* was named *mis1*$^{+}$ (*mis*, *m*inichromosome *in*stability) and it was found that the t.s. *mis1* mutants had an increased frequency of minichromosome loss and elongated cells with a single nucleus at the restrictive temperature (Takahashi *et al.,* 1994). The *cdc1*$^{+}$ gene was cloned (MacNeill *et al.,* 1996) by screening for genes that were able to rescue the *cdc1-P13* mutation identified by Nurse *et al.*, (1976). A diploid *cdc1*$^{+}$/*cdc1Δ* strain was created and it was discovered that the spores containing the deletion were inviable. Hence, *cdc1*$^{+}$ is essential (MacNeill *et al.,* 1996). *cdc1Δ* cells

grown in culture do not increase in cell number and they arrest with a 2C DNA content.

As mentioned above, expression of Cdc1 can rescue t.s. mutations in *cdc1* (MacNeill *et al.,* 1996). Experiments were performed to investigate the regions of Cdc1 that are important for this rescue. This was carried out by investigating the ability of Cdc1 truncations to rescue the t.s. strain *cdc1-P13* (MacNeill *et al.,* 1996). Deletion of the first 25 or 50 amino acids of Cdc1 causes an inability of the truncated protein to rescue the t.s. strain. A similar inability to rescue is seen with a deletion of the last 20 or 30 amino acids. On the other hand, deletion of the last ten amino acids does not affect the ability of Cdc1 to rescue the t.s. strain, it can rescue as well as the full length protein. This suggests that the first 50 amino acids of Cdc1 and the amino acids between the last 20 and 30 might be important for the function of Cdc1.

In *S. cerevisiae* the B subunit of polδ is Pol31. Pol31 was identified in a screen that looked for mutants that were hypersensitive to hydroxyurea, and was called *hys2* (*hy*droxyurea *s*ensitive; (Sugimoto *et al.,* 1995). The *hys2-1* mutant was found to, at the restrictive temperature, arrest with large buds with a single nucleus located at the proximity of the neck that separates the mother and daughter cells, and a short spindle, consistent with mutants that are deficient in either mitosis or DNA replication (Sugimoto *et al.,* 1995). *hys2-1* was also found to arrest with a 2C DNA content although the DNA was not replicated correctly as indicated by pulse field gel electrophoresis. Cloning of the $hys2^{+}$ gene and its deletion have shown that it is essential. It was then discovered that Hys2 was the B subunit of polδ (Maki *et al.,* 1998) as it co-purified with the A subunit and polδ polymerase activity. *S. cerevisiae*'s B subunit has also been identified in a screen for mutations that rescued t.s. mutation of the catalytic subunit. In this screen the B subunit was named *sdp5* (Giot *et al.,* 1997). The name Pol31 instead of Hys2/Sdp5 was suggested (Gerik *et al.,* 1998) indicating that it is the second subunit of polδ (Pol3 being the first and Pol32 the third).

Homologues of Cdc1 share a significant degree of homology. Cdc1 and Pol31 are 33% identical (MacNeill *et al.,* 1996); Cdc1 and its *Xenopus laevis* counterpart (XlCdc1) share 34% identity (Reynolds and MacNeill, 1999). The B subunit is widely conserved. As well as the above mentioned organisms Cdc1 homologues have been found in every eukaryote that has been investigated. Further, Cdc1 homologues have been found in archaea (Makiniemi *et al.,* 1999) associated with proteins that display DNA polymerase and exonuclease activities ((Uemori *et al.,* 1997); see section 1.6 for a discussion on archaeal DNA replication). Since the B subunit is found in both archaea and eukarya it is more widely conserved than the catalytic (A) subunit. This degree of conservation between species prompted the investigation of whether the B subunits were interchangeable between species (MacNeill *et al.,* 1996). It is known that expression of Cdc1 can rescue t.s. mutations of *cdc1*, see above. However, when Pol31 was investigated to see if it had the same effect on the *cdc1-P13* mutation it was found that it did not and that Pol31 cannot rescue a t.s. mutation on *cdc1*. Furthermore, Cdc1 cannot rescue mutations in *pol31* (MacNeill *et al.,* 1996). Figure 1.3 below shows a sequence alignment of the different polδ B subunits in a wide range of organisms.

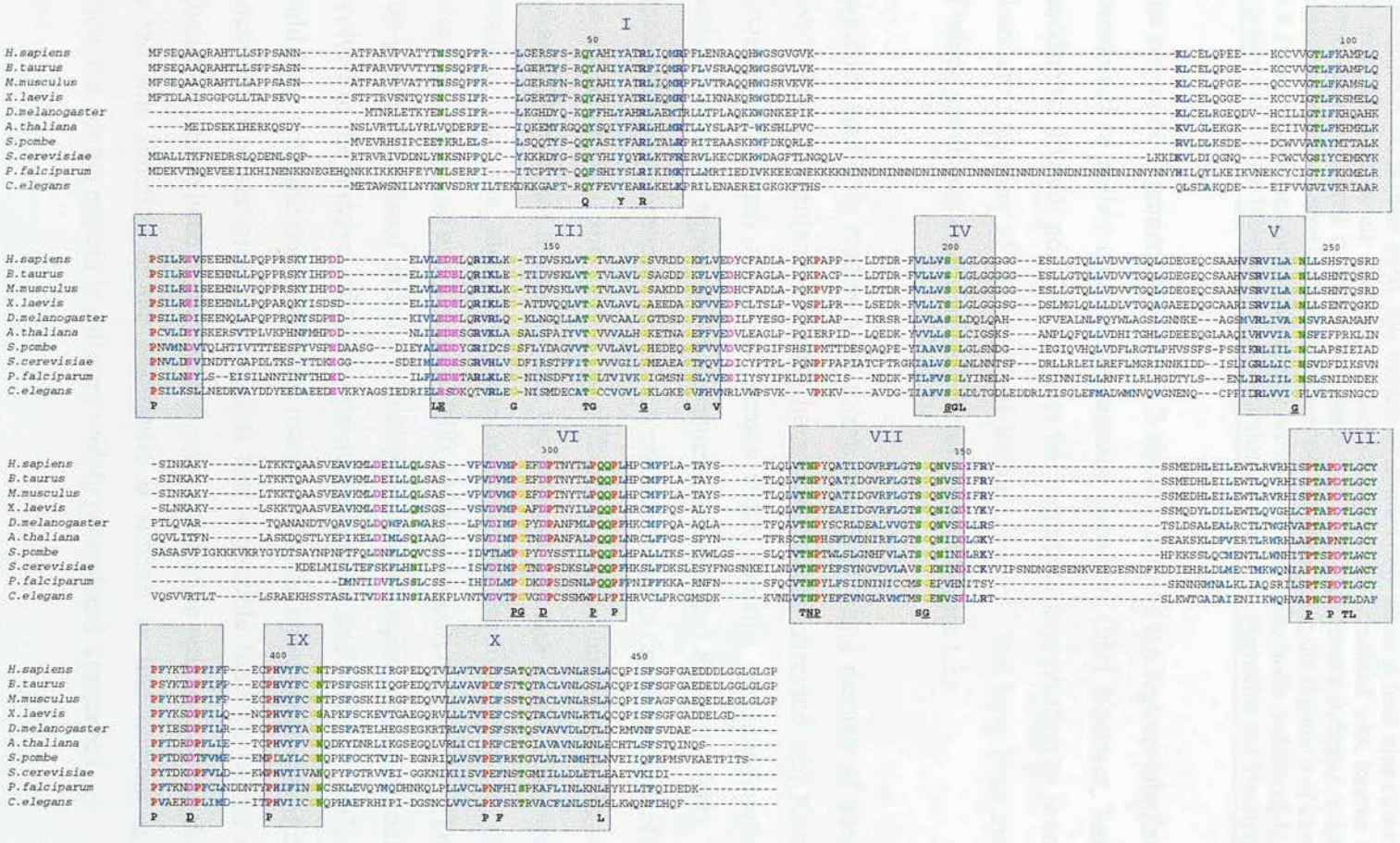
I
II
III
IV
V
VI
VII
VIII
IX
X
H.sapiens
B.taurus
M.musculus
X.laevis
D.melanogaster
A.thaliana
S.pombe
S.cerevisiae
P.falciparum
C.elegans

Figure 1.3. Protein alignment of B subunit proteins from ten eukaryotic species. Sequences are shown in single letter code. Colour scheme: G (yellow), P (red), hydrophobic side chains L, I, V, A, M, F, Y (light blue), polar side chains S, T, N, Q, C (green), acidic side chains D and E (magenta), and basic side chains K, R, H (dark blue). The colours are applied only where at least nine of the ten sequences are from the same colour grouping. Conserved regions are identified with Roman numerals I-X. Amino acids conserved in all ten eukaryotic B subunit proteins are indicated below the aligned sequences; residues conserved across archaeal DP1 proteins (using the alignment of Cann *et al.* (1998) as a guide, but with minor modifications) are underlined. Amino acids underlined in the *S. pombe* sequence indicate the location of three t.s. strains. Adapted from (Reynolds and MacNeill, 1999).

The sequence alignment in Figure 1.3 above identified ten regions labelled I to X that correspond to highly conserved sequence stretches. Cdc1 however, has no known function so it is not possible to relate the regions of conservation to functional sites. There are a number of t.s. mutations isolated in *cdc1*$^{+}$ that have been mapped, three of which are shown as underlined amino acids in Figure 1.3.

Sequence similarity studies have shown that Cdc1 has a number of domains which have sequence similarity to phosphoesterase motifs (Aravind and Koonin, 1998). However, it appears that in *S. pombe* these domains are non-functional as the catalytic domains appear to be disrupted (Aravind and Koonin, 1998). These non-functional phosphoesterase domains were also found in the B subunits of polα, δ and ε in all eukaryotes, and also in the archaeal B subunit homologue. However, in the archaeal homologue it appears that the catalytic domain is not disrupted, implying therefore, that the phosphoesterase motif might be functional in the archaeal homologue (Aravind and Koonin, 1998). During the polymerisation of DNA there is a by-product produced called pyrophosphate, the phosphoesterase domain could be involved in the removal of this by-product (Aravind and Koonin, 1998) and thus it could help with the polymerisation reaction. As the phosphoesterase domain is not functional in eukaryotes, this suggests that a possible function of the ancestral B subunit may have been to help with the polymerisation reaction, although this cannot be the sole function of the eukaryotic B subunit as this domain is no longer functional yet the protein is still very widely present and conserved.

The mammalian polδ B subunit (PolD2) binds to Wrn, the Werner Syndrome protein. Werner syndrome is a syndrome that resembles premature ageing (Szekely *et al.,* 2000). The *S. pombe* polδ B subunit (Cdc1) binds to Dna2, a protein involved in Okazaki fragment processing (Kang *et al.,* 2000). Hence, it appears that a possible function of polδ B subunit could be in interacting with other proteins for the localisation of polδ. These two interactions will be discussed in more depth in section 1.3.3.2.2.

1.3.3.1.3 Cdc27

Cdc27, the C subunit, is an essential protein of 372 amino acids which, although it has a predicted size of 43 kDa (Hughes *et al.,* 1992), migrates as a protein of 54 kDa in size (Zuo *et al.,* 1997). As with *pol3*$^+$ and *cdc1*$^+$, *cdc27*$^+$ was first defined in a similar screen that attempted to identify *cdc* mutants (Nasmyth and Nurse, 1981). The *cdc27* mutant was found to arrest at the restrictive temperature of 36°C with highly elongated cells, indicative of a defect in either DNA synthesis or nuclear division (described in Nurse *et al.,* 1976). As well as the elongated cells, the *cdc27* mutant cells also showed a single nucleus and a 2C DNA content. From the determination of the transition point (Nasmyth and Nurse, 1981) it was first thought that *cdc27* was involved in nuclear division. The *cdc27*$^+$ gene was cloned by screening for suppressors of a *cdc27* t.s. mutant identified in the above screen (Hughes *et al.,* 1992). This subunit has a PCNA binding consensus sequence (Q-XX-I-XX-FF) located at its C-terminus, and therefore, likely binds to PCNA ((Reynolds *et al.,* 2000b); see section 1.3.4.1 for a discussion of PCNA). Further, it is involved in the binding to Cdc1 and it was found to be able to bind to Cdc1 and PCNA at the same time (Reynolds *et al.,* 2000b).

In *S. cerevisiae* the homologue of Cdc27 is Pol32, the 55 kDa product of the *pol32*$^+$ gene, and they share 20% sequence identity (Gerik *et al.,* 1998). Even though Cdc27 is essential it was found that a deletion of Pol32 was not lethal (Gerik *et al.,* 1998). This *pol32Δ* was however, found to be: cold sensitive; hypersensitive to hydroxyurea (an inhibitor of replication) and lethal in combination with other mutants in polδ. As

with its *S. pombe* homologue, Pol32 has a PCNA binding motif located in its C-terminus and has been found to be involved in direct interactions with PCNA (Gerik *et al.,* 1998). Some *S. cerevisiae* polδ complexes lacking the C- subunit have been found (Burgers and Gerik, 1998). This two subunit complex however, was found to need a much higher concentration of PCNA, when compared to the three subunit complex, to be processive. Further, it was found to pause much more often when compared to the three subunit complex (Burgers and Gerik, 1998). These defects are consistent with impaired PCNA-polδ binding to the two subunit complex. As well as the interaction with PCNA it was also found that the C subunits of both *S. pombe* and *S. cerevisiae* polδ are able to bind to the catalytic subunit of polα (Huang *et al.,* 1999c and S. MacNeill, unpublished results). This is interesting as it is suggestive of possible interaction between different polymerases at the replication fork. It also appears that the C subunit is involved in the binding to some helicases (see section 1.3.3.2.2).

At first it was thought that the mammalian polδ did not have a C subunit. However, Hughes *et al.*, (1999b) performed PCNA binding chromatography and found that as well as polδ A and B subunits, another polypeptide was associated, which was called p66. This was found to be encoded by the putative gene KIAA0039. The protein produced was found to have very low but significant homology with Cdc27 and Pol32. In the C- terminus of the proteins this homology was found to be 32.4% identical (Hughes *et al.,* 1999b). Further, p66, as well as Cdc27 and Pol32, was found to contain a PCNA-binding motif in its C- terminus. In similar screens to that by Hughes *et al.*, (1999b) the C subunit from polδ in calf thymus (Mo *et al.,* 2000) and from human cell extracts (Shikata *et al.,* 2001) have been identified. Human p66 was found to stabilise the interaction found between the A and the B subunit (Shikata *et al.,* 2001). It was also found that even though both the human A and C subunits are able to interact with PCNA the presence of human p66 in the complex appears to cause PCNA to bind with a greater affinity to polδ (Shikata *et al.,* 2001).

It appears that the function of the C subunit of polδ is to interact with PCNA (and sometimes to increase the affinity of this binding), and that it is involved in the

binding to polα and could be involved in the stabilisation of the A and B subunit interaction.

1.3.3.1.4 Cdm1

Cdm1, the D subunit, is the smallest component of the complex, being only 22 kDa in size. It was first identified as a suppressor of the *cdc1-P13* mutation (MacNeill *et al.,* 1996), from a *S. pombe* genomic DNA library. When polδ was purified it was found that Cdm1 was in fact a member of the polδ complex as it was associated with the other subunits (Pol3, Cdc1 and Cdc27; (Zuo *et al.,* 1997)).

Purification of mammalian polδ by Liu *et al.*, (2000a) demonstrated that this enzyme had four subunits as opposed to most previous purifications that only identified two subunits. One of the subunits identified, p12, was a 12 kDa protein that was found to be the mammalian homologue of Cdm1 as it has a small but significant homology to Cdm1 (Liu *et al.,* 2000a). p12 and Cdm1 share 25% identity, however, in the C-terminal region the identity increases to 44% (Liu *et al.,* 2000a). Interestingly, it appears that there is no homologue of the D subunit present in *S. cerevisiae.* Purification of *S. cerevisiae* polδ by Eissenberg *et al.*, (1997) does not identify a fourth subunit and a homologue of the D subunit cannot be found by database searches (Liu *et al.,* 2000a). This however, could mean that the D subunit in *S. cerevisiae* does not co-purify easily maybe because of being prone to degradation. The inability to find the *S. cerevisiae* homologue in a database search might simply mean that this homologue is more distant than Cdm1 and p12 are to each other. Hence, *S. cerevisiae* might have a D subunit but it has not been possible to identify it as yet.

Cdm1 is not essential, as deletion of this gene in *S. pombe* produced cells that had DNA repair and DNA replication phenotypes that were indistinguishable from wild-type (Reynolds *et al.,* 1998). Overexpression of Cdm1 is however, able to rescue t.s. mutations in the other members of the complex (Reynolds *et al.,* 1998). Also, Cdm1 was found to be needed for the formation of the polδ complex: expression of Pol3,

Cdc1 and Cdc27 (without Cdm1) in insect cells failed to produce soluble polδ and attempts to purify polδ from cells deleted for Cdm1 where unsuccessful (Zuo *et al.*, 2000c). Furthermore, the human homologue of Cdm1, p12 was found to stimulate the polymerase activity of polδ (Podust *et al.*, 2002).

1.3.3.2 Interactions of polδ

1.3.3.2.1 Interactions within polδ

The interactions of the polδ complex are summarised in Figure 1.2 above. In brief, Pol3 is able to interact with Cdc1 and Cdc1 with Cdc27, but an interaction between Pol3 and Cdc27 has never been shown. The interactions of Cdm1 in the complex are not known.

Pol3 is known to bind to Cdc1. This was first discovered in a two-hybrid assay performed with Pol3 (from which the first 211 amino acids had been deleted) and Cdc1, and was later confirmed by *in vitro* studies (MacNeill *et al.*, 1996). The precise regions involved in the interaction between Pol3 and Cdc1 are unknown, however, some evidence suggests it is via a putative zinc finger region located in the C- terminus of Pol3. Certain mutations in the zinc finger regions of Pol3 in *S. cerevisiae* can be rescued if a B subunit mutant is overexpressed (Giot *et al.*, 1995; Giot *et al.*, 1997). Further, in polε a deletion in the zinc finger region of the catalytic subunit causes binding to the B subunit to be abolished (Dua *et al.*, 2000), although, this could also be due to an inability of the catalytic subunit to fold properly or to it being degraded. Finally, in mouse polα a 230 amino acid region that contains a zinc finger region is able to bind to the accessory subunit (Mizuno *et al.*, 1999). Therefore, it is possible that in *S. pombe* the interaction of Pol3 and Cdc1 is mediated via the putative zinc finger region of the C-terminus of Pol3.

Cdc1 is involved in binding to both Pol3 and Cdc27, as was shown by two-hybrid and *in vitro* studies (MacNeill *et al.*, 1996), but the precise regions of Cdc1 involved in this binding are not clear. Two-hybrid analysis has shown that deletion of the N-

terminal 25 amino acids of Cdc1 does not affect binding to Cdc27, whereas deletion of the N- terminal 50 amino acids abolishes it (MacNeill *et al.,* 1996). Also, deletion of the C- terminal 10 amino acids does not affect binding to Cdc27, but deletion of the C- terminal 20 or 30 amino acids abolishes it (MacNeill *et al.,* 1996). This suggests that both the N and C- termini of Cdc1 are important for binding to Cdc27. However, it is possible that the inability of the truncations to bind could be due to an effect on the overall stability or structure of the protein and not a direct effect on binding. Also, a two-hybrid screen, which used Cdc27 as bait, was carried out and a fragment of Cdc1 was found to interact. This Cdc1 fragment was found to be comprised only of amino acids 157 to 462, which would suggest that the binding of Cdc1 to Cdc27 may be via this region of Cdc1 (E. Murray-Smith, this lab, unpublished results).

The region of Cdc27 involved in binding to Cdc1 has been investigated by Reynolds *et al.* (2000b). A series of truncated Cdc27 proteins were constructed, to be tested in the two-hybrid system. It was found that the first 160 amino acids of Cdc27 are required for binding to Cdc1. This was also confirmed by *in vitro* studies using GST-tagged Cdc27. Therefore, binding of Cdc27 to Cdc1 is mediated by the first 160 amino acids of Cdc27.

The interactions of the fourth and smallest subunit of the complex, Cdm1, are not known. A study by Reynolds *et al.* (2000b) has shown that genetically $cdm1^+$ is able to interact with all of the subunits of the complex, as over-expression of $cdm1^+$ is able to rescue t.s. mutations in *pol3*, *cdc1* and *cdc27*. Work by Zuo *et al.*, (2000c) has shown that if the three subunits, Pol3, Cdc1 and Cdm1 are expressed from insect cells they are able to form a complex that has polymerase activity. This suggests that Cdm1 is able to interact with either Pol3 or Cdc1. It however does not rule out the possibility that Cdm1 might also be able to interact with Cdc27. Nevertheless, at present there is no evidence for direct interactions between Cdm1 and any of the other components of polδ.

1.3.3.2.2 Interactions outwith polδ

Polδ is active at the replication fork where there are a large number of proteins involved in DNA replication, some of which are able to interact with each other. At the fork, one of the proteins that is associated with polδ is the Proliferating Cell Nuclear Antigen, PCNA. This protein tethers polδ to the DNA and gives it its high processivity (see section 1.3.4.1 for a discussion of PCNA). The binding of PCNA with polδ is mediated via Cdc27, the C subunit. A PCNA binding motif was found in the extreme C-terminus of Cdc27, and a 20 amino acid fragment corresponding to this region alone is able to bind to PCNA both *in vitro* and in the two-hybrid system (Reynolds *et al.,* 2000b). Interestingly, there is some evidence that suggests that the catalytic subunit of mammalian polδ might be involved directly in binding to PCNA. This was suggested from the following evidence: a peptide to a region of human polδ (N2) can inhibit the stimulation of polδ by PCNA, and can bind to PCNA as shown by dot blot experiments (Zhang *et al.,* 1995). It was also shown that PCNA and the A subunit are able to interact by their co-immunoprecipitation in human (Shikata *et al.,* 2001) and bovine cell extracts, cross linking experiments and further dot blot analysis (Zhang *et al.,* 1999). However, for *S. pombe* it was shown by co-immunoprecipitation and a two-hybrid assay that this is not the case (Tratner *et al.,* 1997). Further, in *S. cerevisiae* interactions of polδ with PCNA were investigated by running polδ on a gel and blotting with PCNA and it was found that the only subunit that is able to bind to PCNA is the C subunit, Pol32. In calf thymus, recent work has demonstrated an interaction between the B subunit and PCNA via a hydrophobic five amino acid region (Lu *et al.,* 2002). This has been shown in immunoprecipitation and binding studies with a 22 amino acid peptide containing the five amino acids thought to be involved in the binding. Further, it was demonstrated that PCNA did not interact with the A subunit (Lu *et al.,* 2002). Thus, there is evidence to suggest that the subunit which interacts with PCNA may vary depending on the organism studied or on the methods adopted to study it. PCNA could interact with the A, B or C subunit. Further work specifically in mammalian systems might help to elucidate this.

Polδ is also able to interact with the catalytic subunit of polα. This interaction was discovered by Huang *et al.* (1999c) after a two-hybrid screen with the *S. cerevisiae* homologue of Cdc27, Pol32, was carried out. In the assay a number of clones of polα were found to interact with Pol32, the smallest of which corresponds to a fragment from amino acid 313 to amino acid 533. Recent studies have shown that this is also the case in *S. pombe* where Cdc27 is able to interact with Pol1, the catalytic subunit of *S. pombe* polα (S. MacNeill, unpublished results). This interaction between polδ and polα is very interesting as it suggests that these two polymerases are able to interact, probably at the replication fork, during replication. Furthermore, it is tempting to suggest that this interaction may have a biological significance in co-ordinating both enzymes.

Werner syndrome, in humans, is a syndrome that resembles premature ageing and is caused by the inactivation of the *WRN* gene which encodes a helicase. The symptoms of Werner syndrome include premature greying and thinning of hair, calcification of heart valves, osteoporosis, tumours and the average age of death is 47 years (Martin, 1997). Cells with Werner syndrome differ from normal ageing cells in that Werner syndrome cells have an elongated S phase and do not have shortened telomeres (Martin, 1997). In a two-hybrid analysis it was found that the C- terminus of the Wrn protein was able to bind to the human homologue of the polδ B subunit (Szekely *et al.,* 2000). This interaction was confirmed by immunoprecipitation studies. Furthermore, it was found that WRN recruits the B subunit to the nucleolus and that the B subunit is still able to bind to the A subunit. In addition, the polymerase activity of *S. cerevisiae* polδ can be stimulated *in vitro* by addition of human Wrn protein. This suggests that the B subunit might have a function in the localisation of polδ. The effect of the human Wrn protein on *S. cerevisiae* polδ has been investigated (Kamath-Loeb *et al.,* 2000). It was found that in the absence of PCNA Wrn was able to stimulate polymerase activity of polδ. This stimulation depended on the presence of the C subunit, Pol32. Further, in the presence of PCNA the stimulation by Wrn was not seen (Kamath-Loeb *et al.,* 2000).

In *S. pombe* the *WRN* homologue is *rqh1*, it appears to encode a helicase, like *wrn*, and it appears to be involved in DNA repair (Murray *et al.,* 1997). It is thought to be involved in the recombination functions that happen during arrests in S-phase. Interestingly, Rqh1 is required where mutations that affect the elongation phase of DNA replication (like Cdc1) are present but not with mutants that affect the initiation of S phase (Murray *et al.,* 1997). Attempts to find a direct interaction, via the two-hybrid system, of Rqh1 and Cdc1 have so far failed (S. MacNeill, personal communication).

An interaction between bovine polδ and another helicase was also seen (Carastro *et al.,* 2002). The helicase is the bovine homologue of the human helicase HUPF1. It is thought to have 5' to 3' helicase activity and can function on either DNA or RNA. This helicase was shown to be able to interact with the C subunit of bovine polδ.

Originally it was thought that polδ was able to dimerise. The evidence for this came from two-hybrid interactions of the *S. cerevisiae* C subunit with itself. Furthermore, it was found that the C subunit, when bound to the B subunit was able to dimerise *in vitro* (Gerik *et al.,* 1998). This was confirmed by the apparent size of the polδ complex in *S. cerevisiae*, which corresponded to it being a dimer (Burgers and Gerik, 1998). The dimeric state of polδ would provide further weight to the hypothesis that it can synthesise both leading and lagging strand DNA as this interaction would probably be present at the replication fork, while synthesising DNA. However, recent studies of the stoichiometry and structure of polδ have shown that the reason why polδ appeared to be a dimer is because of the shape of the C subunit. The C subunit has an unusually elongated shape and it causes polδ to have an overall elongated shape as well, therefore, it appears to be larger than would be expected for a monomer (Johansson *et al.,* 2001). Furthermore, it was discovered that the two-hybrid interaction between the two C subunits, described above, was most likely due to a bridging effect of PCNA. In *S. pombe* it was also thought that polδ was a dimer, as suggested by gel filtration studies (Zuo *et al.,* 2000c). However, it has recently been shown that *S. pombe* Cdc27, like *S. cerevisiae* Pol32, is elongated and polδ is in

actual fact a monomer (Bermudez *et al.*, 2002). Therefore, it appears that polδ is not able to dimerise.

Cdc24 is a protein in *S. pombe* that is essential for the completion of S phase. No homologues have been found in any other organism but Cdc24 has been shown to interact with DNA replication proteins. It is able to interact genetically with *rfc1*, *pcn1*, *cdm1* and *dna2*, as overexpression of Cdc24 can rescue some mutations in the above genes. Furthermore, it has been shown to interact in a two-hybrid assay with Dna2 and it has been co-immunoprecipitated with Rfc1 and Pcn1. A screen was done by H. Tanaka (unpublished data) to identify cold sensitive suppressors of *cdc24-M38*. The *cdc24-M38* is a t.s. mutation that has amino acid 370(Arg) mutated to a stop codon, creating a truncated protein (wild type is 501 amino acids in length). The screen identified, amongst other proteins, a mutation in Pol3 and also, mutations in Cdc27, which were able to suppress the mutation in Cdc24. The Pol3 mutation was not determined, but it was shown that all of the Cdc27 mutants that were able to rescue the Cdc24 mutation were C- terminal truncations. There were five Cdc27 mutants isolated in the screen, all of differing length. They were 164, 175, 179, 245, and 271 amino acids in length whereas wild type Cdc27 is 372 amino acids long. Polδ, via Cdc27, is therefore able to interact genetically with Cdc24.

Dna2 is a protein involved in Okazaki fragment processing (see section 1.5, for a discussion of Okazaki fragment maturation). It was found that t.s. mutations of Dna2 could be rescued by Cdc27 and Cdc1 (Kang *et al.*, 2000). It was then confirmed by two-hybrid studies that Cdc1 was able to interact with Dna2, but these studies also showed that Cdc27 had very weak, transient interactions with Dna2. Therefore, it appears that Dna2 is able to interact with polδ and it might have a role in tethering polδ for Okazaki fragment processing.

1.3.4 Accessory Factors to DNA Polymerases

DNA polymerases do not work alone. There are many factors involved in DNA replication, such as PCNA and RF-C.

1.3.4.1 Proliferating Cell Nuclear Antigen

Proliferating Cell Nuclear Antigen (PCNA) was identified by Miyachi *et al.* (1978) who identified it in the nucleus of cells from patients with systemic lupus erythematosus. It was also identified as cyclin, a protein whose expression level increased in S- phase (not to be confused with cdc2; (Bravo and Celis, 1980)). Later it was found that cyclin and PCNA were the same protein (Mathews *et al.,* 1984). Subsequently PCNA was found to be required for efficient DNA replication in the SV40 model system (Prelich *et al.,* 1987) as extracts lacking PCNA did not complete replication after a short burst of replication. Addition of PCNA to these extracts resulted in the ability of the extract to perform efficient DNA synthesis (Prelich *et al.,* 1987). Further, it was found that these extracts that lack PCNA can only synthesise short DNA fragments that correspond to Okazaki fragments (Prelich and Stillman, 1988) suggesting that PCNA is required for leading strand synthesis. Hence, it was suggested that PCNA is specifically needed for co-ordinated leading and lagging strand synthesis (Prelich and Stillman, 1988).

PCNA, also called the sliding clamp, is a homotrimeric molecule of toroidal shape (Krishna *et al.,* 1994), which is remarkably similar to the *E. coli* sliding clamp (Kong *et al.,* 1992). The structure has a hole in the middle which has a large enough diameter for two strands of DNA and the associated water molecules to fit through. This structure allows PCNA to act as a sliding clamp, holding the DNA polymerase in position close to the DNA molecule, but still being able to move along it. Polδ alone is not a processive enzyme, but in association with PCNA it is highly processive. This interaction is mediated through the C subunit, Cdc27 (see section 1.3.3.1.3). By contrast, Polε is processive in the absence of PCNA but it nonetheless associates with it, which stimulates its processivity (see section 1.3.2; (Dua *et al.,* 2002)).

The regions of PCNA involved in the interactions with polδ and polε have been identified by Eissenberg *et al.*, (1997). Two different mutants were identified (*pol30-*

79 and *pol30-90*), that differed in their interactions with polδ and ε. *pol30-79* is a mutation of two amino acids that are very conserved in PCNA, and they are located in the loop motif, in a hydrophobic cleft where protein-protein interactions would be expected to occur. This mutant was found to be able to bind to polε but not to polδ, specifically the mutant was defective in the interaction with Pol32, the C- subunit, that contains a PCNA binding motif ((Eissenberg *et al.,* 1997); see section 1.3.3.1.3). *pol30-90* is a mutation of two amino acids in the C- terminus of PCNA. It was found that this mutant is able to bind to polδ but not to polε (Eissenberg *et al.,* 1997). Therefore, it is thought that polδ and polε interact with PCNA via two different sites: polδ interacts via the loop motif in PCNA and polε interacts via the C- terminus of PCNA (Eissenberg *et al.,* 1997).

The structure of PCNA is closed, it will encircle the DNA until it is either released or until, in the case of linear molecules, it falls off the end. Due to its closed circular structure PCNA needs to be loaded onto the DNA, it cannot simply slide on. It must be opened, encircle the DNA and be closed once again. This loading of PCNA is carried out by replication factor C (RF-C; (Mossi and Hubscher, 1998)).

1.3.4.2 Replication Factor C

Replication Factor C (RF-C) is also called the clamp loader. It is a five subunit complex with homologues found in metazoans, archaea and both *S. cerevisiae* and *S. pombe*. In *S. cerevisiae* and mammals all the subunits have been shown to be essential (Mossi and Hubscher, 1998). The five subunits have a high degree of conservation and they all contain conserved regions II to VIII (Mossi and Hubscher, 1998). RF-C1 is the largest subunit and it contains a conserved region, region I, not present in the other subunits. This region has some resemblance to a BRCT domain, which are known to be involved in DNA damage response (Mossi and Hubscher, 1998). Region I of RF-C1 is involved in the binding to DNA although deletion between regions V to VII have shown that these regions are essential for binding. RF-C1 is also involved in the binding to PCNA, as are *S. cerevisiae* RF-C2 and RF-

C3. In the human homologue the subunits involved in the binding to polδ and to polε have been identified: polδ binds to p40 (homologous to *S. cerevisiae* RF-C4) and polε binds to p37 (homologous to *S. cerevisiae* RF-C2). There are two subunits (RF-C2 and RF-C4) that have ATP-binding activity and a few have ATPase activity consistent with the observation that RF-C needs ATP to load PCNA and that the binding of RF-C to PCNA is increased in the presence of ATP (Mossi and Hubscher, 1998). Complex formation of RF-C in humans appears to involve an intermediate complex formed of p36, p37 and p40 (human homologues of *S. cerevisiae* RF-C3, RF-C2, RF-C4, respectively). The large subunit (p140/RF-C1) appears to bind to the complex by the bridging action of p38 (RF-C5; (Mossi and Hubscher, 1998)).

In the presence of PCNA and RPA, RF-C has been found to stimulate the polymerase activity of polδ and polε. The role of this accessory factor in DNA replication is to load the sliding clamp, PCNA, onto the DNA (Baker and Bell, 1998). RF-C is thought to recognise the 5' end of the RNA-DNA primer made by polα and bind to it (Mossi *et al.,* 2000) and it is believed to displace polα by competing for the 3' end of the primer (Maga *et al.,* 2000). Loading of PCNA by RF-C in an ATP-dependent manner then follows. Until recently, it was believed that RF-C then dissociated from the PCNA/DNA complex (Podust *et al.,* 1998) but a recent study suggests that RF-C remains associated with PCNA via RPA (Yuzhakov *et al.,* 1999d). There is evidence to suggest that RF-C is involved in many other cellular processes in addition to its role in DNA replication, such as DNA damage checkpoint (Green *et al.,* 2000) and sister chromatid cohesion (Mayer *et al.,* 2001).

1.3.4.3 Replication Protein A

Replication protein A (RPA) is also called Replication factor A (RF-A; (reviewed in Iftode *et al.,* 1999)). It is an eukaryotic single stranded DNA binding protein, first identified as a protein that was essential for DNA replication in the SV40 system. In humans RPA is formed of three subunits, RPA1, RPA2 and RPA3, and homologues of these subunits have been found in eukaryotes investigated (e.g. *S. cerevisiae*, *S. pombe*, *X. laevis*). RPA has been shown to bind to both ssDNA and to dsDNA

although the binding to ssDNA is much stronger. This DNA binding is mediated by the presence of DNA binding domains in the RPA subunits: RPA1 has three DNA binding domains, RPA2 has one, whereas RPA3 has no DNA binding domains. The mechanism of DNA binding involves two steps. First, after DNA is unwound by a helicase, two DNA binding domains of RPA1 bind unstably to 8nt of ssDNA, then the RPA aligns along the DNA, which causes the other binding domains to be in contact with the DNA and, as a result, RPA binds stably to 30nt of DNA. This interaction stabilises the ssDNA. As well as binding to DNA, RPA has been shown to be able to unwind dsDNA. This however, has not been attributed to a helicase activity of RPA but to a helix-destabilising activity. As well as binding and stabilising ssDNA, work by Yuzhakov *et al.* (1999d) has shown that it plays a much more important role in DNA replication. It seems that the co-ordination of proteins at the replication fork is mediated by RPA. Polα, RF-C, and Polδ all compete for binding to RPA and thus the proteins are able to be switched co-ordinately. Also, RPA appears to have many roles in the cell other than in DNA replication (nucleotide and base excision repair, recombination, cell division, regulation of transcription), the proposed function of RPA in these roles has been identified mainly by the ability of RPA to interact with proteins involved in these processes.

1.4 Elongation

Elongation is the step of DNA replication in which the bulk of the DNA is replicated. At this stage DNA origins will be unwound and single stranded DNA will be bound to, and protected by, replication protein A (RPA). Of the three polymerases involved in chromosomal DNA replication (see section 1.3) only polα can begin replicating DNA *de novo*. Polα uses its primase and polymerase activity to generate a short RNA-DNA primer. After the primer has reached 30nt in length, polα is blocked by RF-C (Mossi *et al.*, 2000). RF-C then binds to the 5'end of the primer, for which it has a preference (Mossi *et al.*, 2000). It then competes with polα for the 3'-OH of the primer, displacing polα (Maga *et al.*, 2000). After PCNA is loaded onto the DNA, the blocking by RF-C is abolished, and if polα was the only polymerase present then it would be re-loaded onto the DNA. However, PCNA will bind preferentially to

polδ over polα, so polδ is now loaded onto the replicating fork, allowing processive, error-free DNA synthesis to be carried out. This process of polymerase switching is likely to be co-ordinated by the competitive interactions of polα, RF-C, PCNA and polδ with RPA (Yuzhakov *et al.,* 1999d).

The continuation of DNA replication involves fork progression, during which the polymerases move along, and continue to replicate, the DNA. For this to happen, the double strand of DNA must be unwound by a helicase. It has been shown that MCM proteins have weak helicase activity (Ishimi, 1997), are required for fork progression (Labib *et al.,* 2000) and move along with the replication fork (Leatherwood, 1998), therefore, it is thought likely that the MCM proteins are the replicative helicase.

Replication forks have two strands, both of which are replicated simultaneously. Due to the nature of polymerases however, which can only polymerise DNA in the 5'→3' direction, only one strand, the leading strand, can be replicated continuously. The other strand, called the lagging strand, is synthesised discontinuously in short fragments called Okazaki fragments, (see Figure 1.4 below). As a result the process discussed above (priming by polα, its displacement by RF-C to load PCNA, polδ binding to PCNA and performing DNA synthesis) must occur more frequently in the lagging strand than in the leading strand.

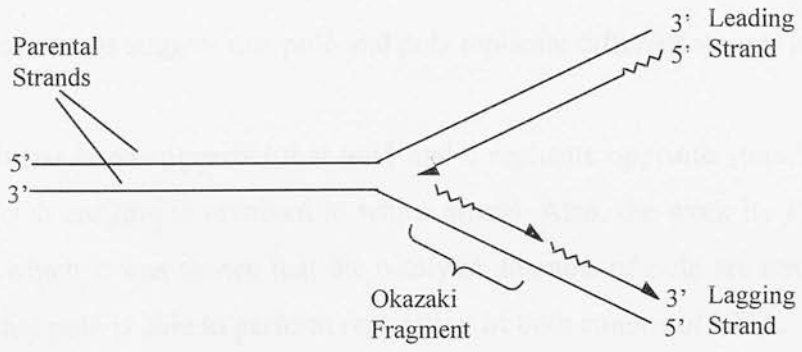

Figure 1.4. DNA is synthesised continuously on one strand (the leading strand). On the other strand (the lagging strand) it is synthesised in non-continuous short Okazaki fragments. Arrows indicate direction of DNA synthesis. Zigzag lines represent the RNA-DNA primer.

Although Figure 1.4 shows DNA being synthesised in opposing physical directions, in actual fact both strands are synthesised in the same physical direction. Twisting of one of the strands (most likely the lagging strand) is needed in order to achieve this. The question arises, are the replicative polymerases strand specific? In other words, is polδ only involved in the polymerisation of one strand and polε in the other? There has been evidence to suggest that pol δ synthesises the leading strand and that pol ε synthesises lagging strand DNA (Burgers, 1991). This is based on the evidence that polε seemed to dissociate from the DNA more readily than polδ and hence it will be suited for lagging strand synthesis (Burgers, 1991). However, it has also been suggested that since polε appears to have inherent processivity it is more suited for the leading strand (Morrison *et al.,* 1990). Work by Karthikeyan *et al.*, (2000d) has shown that polδ and polε replicate different strands at the replication fork. This was shown as follows. The type of DNA sequence has some influence on the types of replication error. By using a template that contained a gene in one orientation and another template that contained the same gene but in a different orientation, different sequences were obtained for the leading and lagging strands in the two orientations. The comparison of the different mutation types and rates of exonuclease deficient (exo^-) polymerases (δ and ε) has shown that the polymerases behave differently with the different orientations of the gene and therefore, that they replicate different sequences for the different orientations of the gene. Also, the polδ exo^- mutant in one gene orientation behaved similarly to polε exo^- in the other orientation. Taken together these results suggest that polδ and polε replicate different strands at the fork.

Therefore, it has been suggested that polδ and ε replicate opposite strands but it is not clear which enzyme is involved in which strand. Also, the work by Kesti *et al.* (1999a), in which it was shown that the catalytic domains of polε are not essential, has shown that polδ is able to perform replication of both strands of DNA.

1.5 Maturation

As described above, the production of an RNA-DNA primer by polα is a necessary step in the initiation of DNA replication. These RNA-DNA primers will have a high frequency of errors as they are synthesised by polα, which does not contain an exonuclease activity (see section 1.3.1). Maturation removes the RNA-DNA primers made by polα/primase and replaces them by DNA synthesised by a proofreading polymerase. Due to the discontinuous nature of lagging strand synthesis, RNA-DNA primers occur at a higher frequency in the lagging strand and so this process is also called Okazaki fragment processing.

In more detail the maturation process is as follows. Polδ which is synthesising an Okazaki fragment reaches the RNA-DNA primer of the following Okazaki fragment. Polδ now brings about strand displacement of the RNA-DNA primer, thus generating a flap. This strand displacement activity is enhanced by the interaction of PCNA with polδ (Maga *et al.,* 2001). The length of the displaced strand is limited by the presence of RPA, which stops polδ after a 30nt flap is generated. This stopping is caused by RPA, which binds with highest affinity to DNA 30nt in length and blocks the polymerase. Interestingly, the length of the primer synthesised by polα is also 30nt, which suggests that RPA will block strand displacement after the whole of the RNA-DNA synthesised by polα is displaced. This ensures that all of the DNA synthesised by the error prone polα is removed. The majority of the flap is removed by Dna2, the remainder of the flap being removed by Fen1 (Bae and Seo, 2000). The apparent preferred substrate for Fen1 is a flap that has an upstream overhang 1nt in length (Kao *et al.,* 2002). Further, if this upstream overhang is longer (as it might be in the case by strand displacement) it appears to inhibit the action of Fen1 (Kao *et al.,* 2002). It is tempting to speculate that the inhibitory effect of a long upstream overhang is to ensure that the flap is long enough to include the DNA synthesised by polα, the error prone DNA polymerase. The action of Dna2 and Fen1 results in a nick that can now be sealed by DNA ligase I. Redundancy exists in this process as it is likely that Dna2 could be replaced by Fen1, and, in cases in which there is no

strand displacement, RNase HI can remove the RNA primer. Thus the RNA-DNA primer is removed and replaced by DNA from a proof reading polymerase.

Dna2, as mentioned before, is involved in the removal of the flap created by the displacement activity of polδ. However, Dna2 has another role in maturation. It has been shown that Dna2 is able to interact genetically with polδ, DNA ligase I and Fen1 (Kang *et al.,* 2000) and this interaction has been confirmed by two-hybrid analysis. Therefore, it appears that Dna2 has another role in which it acts as a core for the localisation of the proteins involved in the maturation processes.

At this stage the DNA has been replicated. The RNA-DNA primers have been removed, replaced by DNA from a proof reading polymerase (either polδ or ε) and nicks have been sealed by DNA ligase. Thus, DNA replication is complete.

1.6 Archaeal DNA Replication

The ongoing sequencing of archaeal genomes, has shed light on this third domain of life (the other two domains being eukarya and prokarya). It appears that eukarya and archaea have a common ancestor that is more recent than the common ancestor linking bacteria with them (Tye, 2000). Sequence analyses have revealed that archaea and eukarya possess many common features of DNA replication although these are less complex in archaea. In archaea there appears to be no ORC homologue. However, homologues of Cdc6/Cdc18 have been found in archaea (with the exception of *M. jannaschii*, in which no homologues have been found), which appear to function as both ORC and MCM loader (MacNeill, 2001b). In eukaryotes on the other hand, these two functions (ORC and MCM loader) are performed by separate conserved proteins (see section 1.2). Thus, it appears that whereas in archaea the one complex can do two functions, these have been split in eukaryotes into two protein complexes. PCNA, the sliding clamp (see section 1.3.4.1), is also present in archaea. Interestingly however, whereas in eukaryotes PCNA is a homotrimeric protein this appears not to always be the case in archaea. Some archaeal PCNAs are formed of two proteins (*S. solfataricus*) whereas *A. pernix*

appears to encode for three PCNA-like proteins (MacNeill, 2001b). The reason for archaea having more than one PCNA whereas eukaryotes only have one is not clear. In eukaryotic PCNA there are distinct sites for the interaction with polδ and ε ((Eissenberg *et al.,* 1997); see section 1.3.4.1) and archaea have two families of DNA polymerases (see below). Thus, maybe the different PCNA proteins ensure different sites for the interaction with the different polymerases. Other factors such as RPA, RF-C, and MCM proteins are also present in archaea and they share homology with their eukaryotic counterparts (MacNeill, 2001b).

Archaeal polymerases (polBI) were first identified as being members of the B family of DNA polymerases, the same family as eukaryotic polα, δ and ε (Cann and Ishino, 1999). These polymerases are monomeric and have DNA polymerase and exonuclease activities. Later, a new polymerase was found in *P. furiosus* (PolD; (Uemori *et al.,* 1997)) that was formed of two proteins (DP1 and DP2) and had DNA polymerase and exonuclease activities (Uemori *et al.,* 1997; Cann and Ishino, 1999). DP2 appears to be a catalytic subunit, larger that DP1 the small associated subunit of unknown function. The DP2 subunit has no known homology with any of the eukaryotic polymerases, whereas DP1 has homology with the B subunit associated with family B polymerases in eukarya ((Makiniemi *et al.,* 1999); polα, δ and ε amongst others). Interestingly, pol D and its homologues appear to have the (apparently) conserved structure of a large catalytic subunit and a smaller associated subunit of unknown function as found in polα, δ and ε. Pol D was found not to belong to any of the existing families of DNA polymerases, so it was proposed (Cann and Ishino, 1999) that it was to be a member of a new family of DNA polymerases, family D. Homologues of pol D have been found in other archaea (Cann and Ishino, 1999). Thus, it appears that archaea have two distinct families of DNA polymerases: family B and family D. Work remains to be done to fully elucidate the mechanism of DNA replication in archaea but it appears to involve very similar players to those found in eukaryotes.

1.7 *S. pombe* as a model organism

In this thesis, the fission yeast *Schizosaccharomyces pombe* was used as a model organism. As it was originally isolated in African beer it was given the name "pombe", which is Swahili for beer (Wixon, 2002). *S. pombe* is a member of the *Ascomycota* phylum, family *Schizosaccharomycetaceae* (Sipiczki, 2000). It is a unicellular organism with rod shaped cells 5-20μm in length (Mitchison, 1957). *S. pombe*, like higher eukaryotic cells, divides by fission and not (as for *S. cerevisiae*) by budding. It has three chromosomes named I, II and III which are approximately 5.7 Mb, 4.6 Mb and 3.5 Mb in size respectively (Wixon, 2002).

One of the main reasons that *S. pombe* was used as a model organism was due to it being so well suited for cell cycle studies. It was found that its growth was linear and that the size of the cell corresponded to the stage of the cell cycle at which the cell was (Mitchison, 1957). Further, it was possible to use size selection (by, for example, the use of an elutriator, a modified centrifuge that allows selection of cells of the same size) to create synchronous cell cultures (Wixon, 2002). Also, this made the characterisation of mutants involved in the cell cycle easy as the mutants defective in a particular stage of the cell cycle will arrest at a particular size. The cell cycle in *S. pombe* has been the focus of many studies and is now highly characterised (for a review see MacNeill and Nurse, 1997).

S. pombe has both a haploid and a diploid cell cycle (see Figure 1.5) and is able to alternate between the two, depending on the conditions in which it finds itself. Whilst nutrients are plentiful, cells will stay in the haploid mitotic cell cycle. When deprived of nutrients a number of things can happen. *S. pombe* has two mating types h^+ and h^-. If only one of these mating types are present when the cell is starved, then it will enter stationary phase, which it will exit after nutrients are plentiful again. If however, both mating types are present, upon being deprived of nutrients two cells of opposite mating types will conjugate to produce a diploid zygote. If the cell continues being deprived of nutrients then the zygote will undergo meiosis and sporulate to produce four haploid ascospores. When nutrients are plentiful these can germinate to produce haploid cells that will enter the haploid mitotic cell cycle. If,

when the zygote is formed, nutrients become plentiful again the diploid cell will enter the diploid mitotic cell cycle, which it will leave when nutrients become deprived to undergo meiosis and sporulate to produce four haploid ascospores as described above.

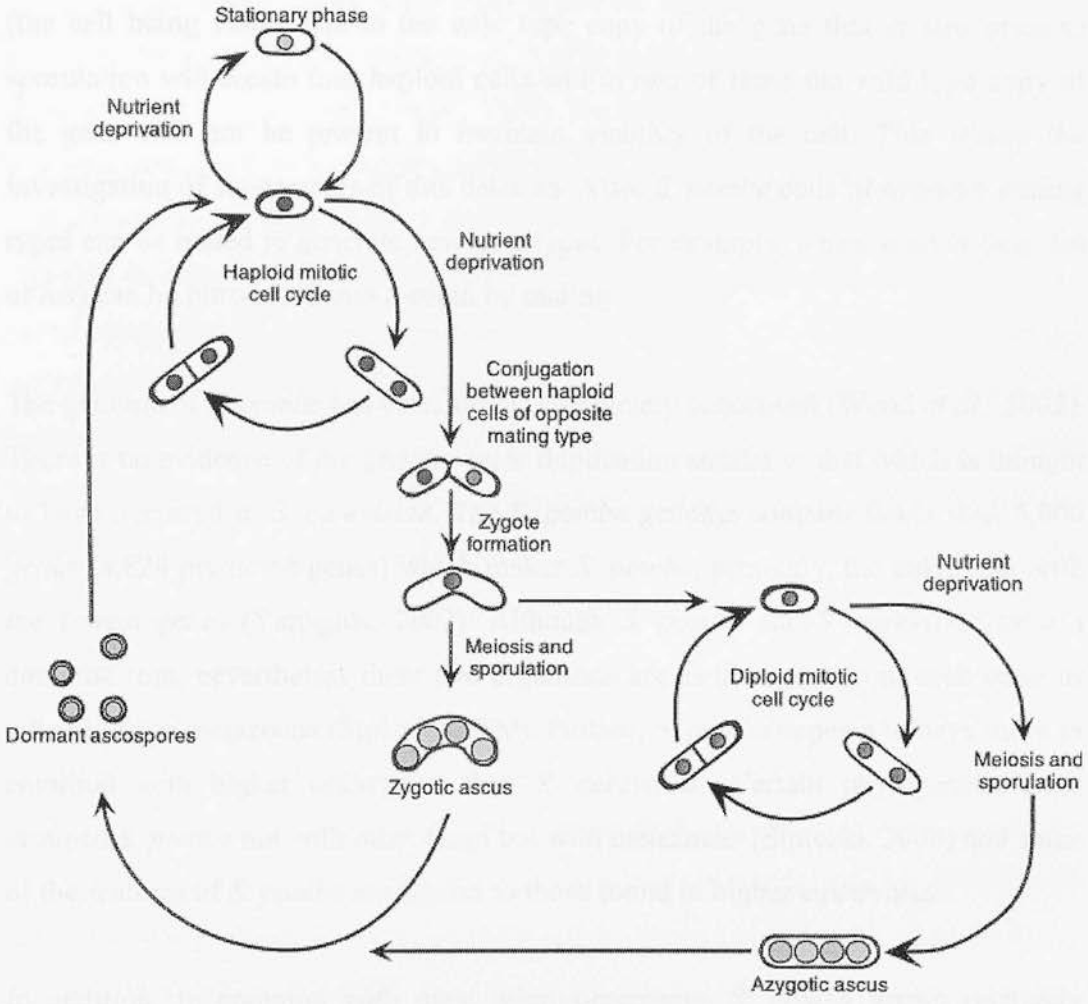

Figure 1.5. Summary of the cell cycles in *S. pombe*. See text for details. Figure courtesy of Dr. S. MacNeill.

The ability of *S. pombe* to alternate between diploid and haploid cell cycles makes possible a number of things. For example, sometimes it is advantageous to create and analyse a deletion of a particular gene. However, if the gene is essential this cannot be done in a haploid organism as the cell will not survive without its essential gene.

In a diploid organism, on the other hand, a deletion of one copy of the essential gene can sometimes be carried out and the cell will remain viable due to the other copy of the gene being present. Also, the transitions between the haploid and diploid cycles are easy to control. By selecting the appropriate media, a diploid strain can be forced to sporulate, resulting in the production of four ascospores that will germinate to produce four haploid cells. Hence, if a deletion gene has been created in a diploid (the cell being viable due to the wild type copy of the gene that is also present) sporulation will create four haploid cells and in two of these the wild type copy of the gene will not be present to maintain viability of the cell. This allows the investigation of suppressors of this deletion. Also, *S. pombe* cells of opposite mating types can be mated to generate new genotypes. For example, a new marker (e.g. *leu* or *his*) can be introduced into a strain by mating.

The genome of *S. pombe* has been almost completely sequenced (Wood *et al.,* 2002). There is no evidence of the genome wide duplication similar to that which is thought to have occurred in *S. cerevisiae*. The *S. pombe* genome contains fewer than 5,000 genes (4,824 predicted genes) which makes *S. pombe*, presently, the eukaryote with the fewest genes (Yanagida, 2002). Although *S. pombe* and *S. cerevisiae* have a common root, nevertheless these two organisms are as divergent from each other as either is from metazoans (Sipiczki, 2000). Further, *S. pombe* appears to have more in common with higher eukaryotes than *S. cerevisiae*. Certain phylogenetic trees grouped *S. pombe* not with other fungi but with metazoans (Sipiczki, 2000) and some of the features of *S. pombe* are similar to those found in higher eukaryotes.

In addition, in common with most micro-organisms, *S. pombe* grows relatively quickly in sterile medium (doubling time of approximately 2.5 hrs at 32°C). Further, as mentioned above several types of media exist that can force *S. pombe* to alternate between the haploid and the diploid life cycles. In summary all these factors described above make *S. pombe* a "small eukaryotic giant" (Yanagida, 2002).

1.8 Aims and Objectives

In *S. pombe* polδ the catalytic activities are located in Pol3 whereas most of the known protein-protein interactions outwith the complex are carried out by Cdc27; for example: binding to PCNA (Reynolds *et al.,* 2000b); binding to the catalytic subunit of polα (S. MacNeill, unpublished results); and genetic interaction with Cdc24 (H. Tanaka, unpublisehd data). What role, therefore, is played by the other subunits? Cdm1 is not essential (Reynolds *et al.,* 1998) and it is not present in *S. cerevisiae* (Liu *et al.,* 2000a; Eissenberg *et al.,* 1997). On the other hand, Cdc1 is essential and is highly conserved with homologues found in all eukaryotic polymerases belonging to the B family and even in the D family of archaeal DNA polymerases. However, its function is unknown. Recent findings suggest that it might be involved in the localisation of polδ via an interaction of the B subunit with Wrn ((Szekely *et al.,* 2000); see section 1.3.3.2.2), however, this experiment was performed using mammalian proteins and the interaction could not be reproduced using *S. pombe* proteins (S. MacNeill, personal communication). It has also been shown to interact with Dna2 and it appears that this interaction is involved in tethering of polδ for Okazaki fragment processing. Hence it appears that Cdc1 could be involved in the localisation of polδ.

In this thesis Cdc1 has been studied. Two approaches were followed: a two-hybrid screen was performed using the B subunit as bait, and an extensive mutational analysis of Cdc1 was carried out. It was hoped that these approaches might identify potential new proteins that interact with Cdc1, or give new information about the binding of Cdc1 within polδ and also that these approaches might yield new information as to the function of Cdc1. As a whole, it was hoped that this work would give further insights into polδ and into the complex process of DNA replication.

2 Pol3-Pol31 Interactions in *S. cerevisiae*

2.1 Introduction

The majority of the known protein-protein interactions of *S. pombe* polδ outwith the complex are carried out by the C subunit, Cdc27. This protein is involved in the binding to PCNA (Reynolds *et al.,* 2000), it is also responsible for binding polδ to polα's catalytic subunit (S. MacNeill, unpublished results). The protein-protein interactions of the B subunit (Cdc1) outwith the complex are not as clear. Recently it was discovered that the mammalian B subunit might be involved in the localisation of polδ to the nucleolus via an interaction with Wrn ((Szekely *et al.,* 2000); see section 1.3.3.2.2). However, the studies that identified the interaction between p50 and Wrn was performed with mammalian proteins and the interaction could not be reproduced using *S. pombe* proteins (Cdc1 and Rqh1; S. MacNeill, personal communication). Also, Cdc1 is thought to help localise polδ to sites of Okazaki fragment processing via an interaction with Dna2 (as demonstrated by two-hybrid analysis; (Kang *et al.,* 2000) see section 1.3.3.2.2).

In order to investigate the protein-protein interactions of Cdc1, a two-hybrid screen was performed. It was hoped that this would identify new interactions that might shed light on the functions of Cdc1. If new interactors were not found it was hoped that this screen might at least help to narrow down the regions involved in the binding to other polδ components. It was also decided not to use Cdc1 but to use Pol31, the *S. cerevisiae* homologue, as this allowed the use of the excellent FRYL budding yeast genomic DNA two-hybrid library (described in section 2.1.2).

2.1.1 Two-hybrid System

The two-hybrid system is a method used to investigate protein interactions between two proteins of interest. The method uses the observation that transcriptional activators are composed of a transcriptional activation domain and a DNA binding domain. The transcriptional activation domain causes activation of the promoter,

whereas the DNA binding domain causes the transcriptional activation domain to be localised at the place where it is needed. The transcriptional activation and DNA binding domains do not need to be fused to each other (Fields and Sternglanz, 1994), in fact, transcriptional activation of the gene can be achieved if the two domains are expressed separately as long as they are still able to interact. In the two-hybrid system, the transcriptional activation domain and the DNA binding domain are separate and cannot directly interact with each other. One of the proteins of interest is fused to the DNA binding domain (this fusion is called the bait protein) and the other protein of interest is fused to the transcriptional activation domain (this fusion is called the prey protein), as shown in Figure 2.1. If the two proteins of interest are able to bind to each other this will cause the transcriptional activation domain to be located close to the promoter due to the indirect interaction with the DNA binding domain. This will cause activation of the reporter gene. As mentioned before, in the absence of the proteins of interest the transcriptional activation domain and the DNA binding domain are unable to interact with each other, hence the activation of the reporter gene can only be caused by the binding of the two proteins of interest to one another. Also, DNA binding domains cannot usually activate the reporter gene on their own, therefore, if the two proteins of interest are not able to interact then the reporter gene will not be activated. See Figure 2.1 below for a summary of the two-hybrid system.

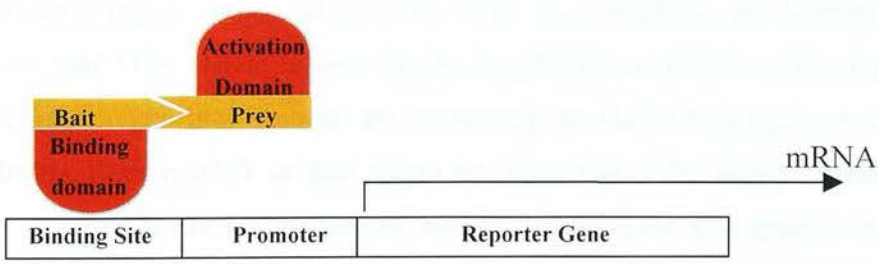

Figure 2.1. Summary of the two-hybrid system. In brief, when the bait and prey fusion proteins are able to interact it causes the Transcriptional Activation Domain to be close to the Promoter causing expression of the reporter gene. The DNA binding domain and the transcriptional activation domain cannot bind to each other in the absence of bait and prey proteins. The reporter gene usually is *HIS3* and/or *LacZ*.

The two-hybrid system allows the study of protein-protein interactions of proteins that are not necessarily endogenous to *S. cerevisiae*. Furthermore, instead of a single protein as prey, a library of fused proteins could be used. This can be used to screen for proteins that interact with the bait.

In this thesis the system used was the *E. coli* LexA DNA binding domain (LexABD) from the pBTM116 plasmid and the *S. cerevisiae* Gal4 transcriptional activation domain (Gal4AD) from either pACT2 or pGAD2F. The reporter genes used were the *HIS3* and *LacZ* genes. The two-hybrid system was used both for screening a library for proteins that can interact with the bait and to test for interactions between the two proteins of interest, Gal4AD-Pol3 and LexABD-Pol31. Three *S. cerevisiae* strains were used to perform two-hybrid analysis: CTY10-5d, L40 and Y187 (see table 6.8). The Y187 contained the FRYL library (see section 2.1.2, below) and it was used by mating to L40, into which a plasmid expressing the LexABD-tagged bait protein was introduced by transformation (see section 6.2.1.9). CTY10-5d was used to investigate the interactions between Gal4AD-Pol3 and LexABD-Pol31.

2.1.2 FRYL library

The FRYL *S. cerevisiae* genomic DNA library was used in this study. This library was made by Micheline Fromont-Racine in Pierre Legrain's lab (Fromont-Racine *et al.*, 1997) by shearing *S. cerevisiae* genomic DNA by sonication and ligating the fragments into pACTIIst, which is very similar to pACT2. pACT2 is a two-hybrid vector, proteins cloned into it will be expressed as Gal4AD-tagged proteins. pACTIIst differs from pACT2 in that it has an additional 17bp linker region, to facilitate the cloning of the library inserts, which contain three stop codons in the three possible reading frames. The library contains a large number of clones, $5x10^6$, with an average size of 700bp and it is expected that an in-frame fusion will occur every 24 bases throughout the genome. Therefore, if a positive interaction is found, it is expected that several independent clones all encoding the same protein will be identified in the screen.

2.2 Results

2.2.1 Two-hybrid mini-screen with Pol31 as bait

The interactions of the B subunit of pol δ with other proteins were investigated in this chapter. For this the two-hybrid system was used, with the FRYL library (described above) as prey, and the B subunit of *S. cerevisiae* polδ (Pol31) as bait (LexABD-Pol31). The pBTM116-Pol31 plasmid was a generous gift of Dr. K. Sugimoto from the University of Nagoya.

Some of the baits are able to self-activate, i.e. they can produce a response from the reporter gene even when they are not bound to a prey protein fused to an transcriptional activation domain. This should be prevented to avoid the identification in the screen of a large number of false positives, proteins that do not interact with the bait protein. This is a problem as it will make harder the identification of proteins that are able to interact with the bait protein. To prevent self-activation of baits the fact that the *HIS* gene is used as a reporter gene is exploited. In the cell the production of histidine can be blocked by 3-amino 1,2,4 Triazole (3-AT), which makes the cell auxotrophic for histidine. This happens as 3-AT can block one step of the pathway used by the cell to produce histidine. Therefore, 3-AT can help alleviate the problem of self-activation, as only cells with sufficiently high expression levels of histidine (i.e. those with interacting proteins, and hence an activated reporter gene) can survive. The concentration of 3-AT needed for a screen varies depending on many factors such as the strain or bait used. In order to find out the appropriate 3-AT concentration a mini-screen is done.

In a mini-screen the 3-AT concentration with which to do a full-screen is determined. The protocol followed is the same as for a full screen, i.e. strains containing bait and prey plasmid (strains L40 and Y187 respectively) are mated on filters, collected, and plated on control plates as well as on a large number of plates (~100) on which only cells that are mated and that have an active reporter gene are able to grow (see

section 6.2.2.3). In a mini-screen, on the other hand, the plating is done on the same control plates but also on plates with varying concentration of 3-AT. In this study the 3-AT concentrations tested were 0 mM, 2.5 mM, 5 mM, 10 mM and in 10 mM intervals until 100 mM. Two plates of each concentration were used. The ideal concentration for a full screen is one in which 1 or 2 colonies grow per plate. This is because in a full screen the number of plates involved is ~100. Hence, 1 or 2 colonies per plate will produce approximately 100-200 colonies, a manageable number of colonies, and these interactions will be more stringent than if a larger number of colonies were to be found per plate. Results suggested that the ideal concentration was 5 mM 3-AT, therefore, this concentration was used to do a full screen with LexABD-Pol31 as bait.

2.2.2 Full two-hybrid screen with Pol31 as bait

A full screen was performed (see section 6.2.2.3 for more details) by mating strains containing plasmids expressing either the bait or the prey plasmids (strains L40 and Y187, respectively). The mated cells were then collected, a sample was removed to be plated onto control plates and the rest of the collected cells were plated in aliquots in plates in which the cells containing positive interacting proteins will be able to form colonies (-LWH+5 mM 3-AT). The growth on the control plates was indicative of the number of diploids screened and the mating efficiency and they were calculated as described in section 6.2.2.3. This screen produced a 31% mating efficiency and 1.39×10^7 diploids were screened. In order to cover the full library the number of diploids screened should be three times that of the expected number of clones in the library, or greater (5×10^6 clones in the library, therefore the number of diploids screened should be 1.5×10^7 or greater). This screen did not fully cover the whole of the library but it was decided to continue nonetheless.

On the –LWH plates 20 colonies grew which were picked and patched onto the same media. Of the 20 colonies patched only 10 grew, the other 10 that did not grow were presumed to be false positives. The cells of these patches were grown in liquid culture from which plasmid rescue was carried out (section 6.2.1.10) and the DNA

obtained from it was used to transform electrocompetent MC1066 *E. coli* cells by electroporation (section 6.2.1.3). Only six of the ten were transformed successfully to produce *E. coli* colonies. These colonies were picked and plasmid DNA was extracted (section 6.2.1.5). The DNA obtained was checked to be pACT by restriction digest and by PCR (sections 6.2.1.17 and 6.2.1.15). The DNA inserts in pACT were sequenced with pACT5', pACT3'and SEQ2HYB (see table 6.2).

Sequencing indicated that the six positives identified in the two-hybrid screen were two different clones, which corresponded to different sized fragments of the C-terminus of Pol3, the catalytic subunit of *S. cerevisiae* DNA polymerase δ. The positive plasmids were called pACT-20, -15, -11, -10, -9, -7. From these plasmids the proteins will be expressed as Gal4AD-tagged proteins. See Figure 2.2 below for a diagrammatic representation of the two-hybrid results.

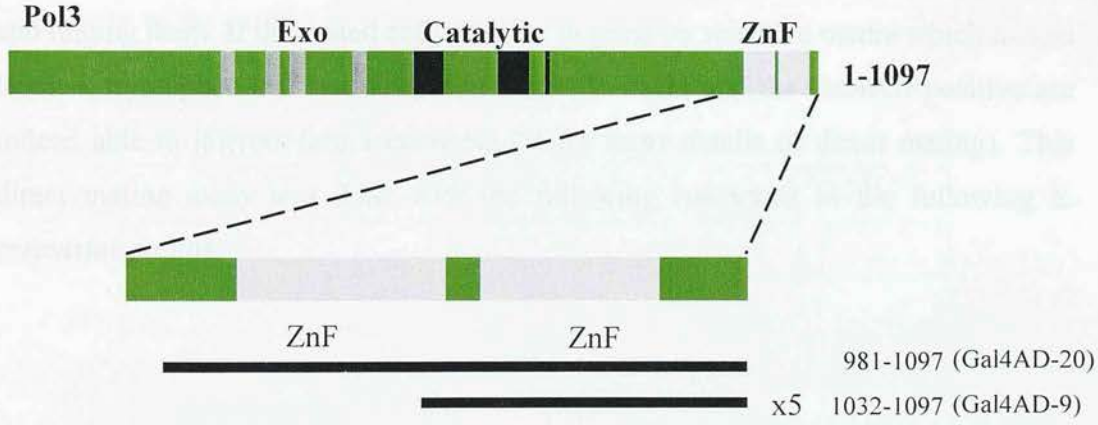

Figure 2.2. Diagram of two-hybrid results. Horizontal black lines indicate the region of the C-terminus of Pol3 obtained from the screen. x5 indicates that the clone was obtained five times. Numbers are amino acid numbers. Exo, exonuclease domains; Catalytic, catalytic domains; ZnF, Zinc Finger.

From the results it is interesting to note that all the positives correspond to overlapping parts of the same protein, Pol3. This suggests that the interaction is real, as it would be unexpected to find different overlapping clones of a false positive. Furthermore, it is interesting to note that the smaller region of binding (Gal4AD-9) is

comprised almost exclusively of the second putative zinc finger, ZnF2. This indicates that this region of Pol3 is sufficient for binding LexABD-Pol31 in the two-hybrid system.

2.2.3 Confirmation of positive clones from two-hybrid screen

The six positives from the two-hybrid screen were checked to ensure that they do interact with LexABD-Pol31. This was done in two ways, 1) direct mating and 2) β-Gal assays.

2.2.3.1 Direct Matings

To check that LexABD-Pol31 and the Gal4AD-tagged positives from the two-hybrid screen interact, direct mating was carried out. In brief, direct mating involves growing an *S. cerevisiae* strain transformed with pBTM116-Pol31, and growing another *S. cerevisiae* strain transformed with the plasmid from one of the positives and mating them. If the mated cells are able to grow on selective media which lacked leucine, tryptophan and histidine, then LexABD-Pol31 and the Gal4AD-positive are indeed able to interact (see section 6.2.2.4 for more details of direct mating). This direct mating assay was done with the following constructs in the following *S. cerevisiae* strains.

Strain	Construct
L40 (a)	pBTM116
	pBTM116-Pol31
Y187 (α)	pACT-20
	pACT-15
	pACT-11
	pACT-10
	pACT-9
	pACT-7
	pACT-Pol3CT

Table 2.1. Plasmids used in direct mating assays and the *S. cerevisiae* strain they were transformed into. pBTM116 produces LexABD-tagged proteins. pACT produces Gal4AD-tagged proteins. pACT-20, -15, -11, -10, -9, and -7 are the positives from the two-hybrid screen. pACT-Pol3CT is a pACT2 plasmid that produces a Gal4AD-tagged *S. pombe* Pol3 clone containing the two C- terminal zinc fingers. "a" or "α" indicate the mating type of the *S. cerevisiae* strain.

The results of the mating are shown in Table 2.2 below.

Control plate (-LW)							
	20	15	11	10	9	7	Pol3CT
LexABD	+	+	+	+	+	+	+
LexABD-Pol31	+	+	+	+	+	+	+

Interaction plate (-LWH)							
	20	15	11	10	9	7	Pol3CT
LexABD	-	-	-	-	-	-	-
LexABD-Pol31	+	+	+	+	+	+	-

Table 2.2. Results of direct mating plates. "+" indicates growth and "-" indicates no growth. Control plate (-LW) is a mating control in which successful mating is shown by the presence of growth. In the interaction plate (-LWH) only positive two-hybrid interactions are able to support growth. Pol3CT is the Gal4AD-tagged *S. pombe* Pol3 clone containing the two C- terminal zinc fingers. 20, 15, 11, 9 and 7 are the Gal4AD-tagged positives from the screen. LexABD is expressed from the empty pBTM116 plasmid.

The control plate shows that all of the matings were successful as indicated by the presence of growth. This is because the mated cells will contain both pBTM116 and pACT plasmids, which confer on the cell the ability to grow on media lacking tryptophan and leucine, respectively. In the interactor plate it can be seen that LexABD-Pol31 is able to interact with all the positives from the screen (Gal4AD-20, -15, -11, -10, -9 and -7). pACT on its own was not tested, however Gal4AD-Pol3CT, the C- terminal region of Pol3 in *S. pombe* containing both putative zinc fingers (clone equivalent to pACT-20) was tested, and the results were negative. This indicates that LexABD-Pol31 does not interact with the Gal4AD tag. pBTM116, which produces the LexABD tag only, is not able to interact with any of the positives from the screen or Pol3CT. This suggests that Gal4AD-20, -15, -11, -10, -9 and -7 are indeed interacting with LexABD-Pol31.

2.2.3.2 Liquid Culture β- Gal Assays

To confirm the results of the library screen and the direct matings the following assay was also done. As mentioned before, when the prey and bait proteins are able to interact, a reporter gene is activated. Up to now, the reporter gene used to test for interactions was the *HIS3* gene. However, there is a second reporter gene used in this system, the *lacZ* gene. This assay exploits the ability of the *lacZ* gene product (β-galactosidase) to metabolise *o*-nitrophenyl-beta-D-galactopyranoside (ONPG) to produce a product with a yellow colour. Furthermore, the intensity of the colour will be proportional to the strength of the interaction as the stronger the interaction the stronger the transcription of the reporter gene. Therefore, this assay could not only provide confirmation of the results shown previously but could also give information about how well the bait and prey are able to interact.

The assay is described in detail in section 6.2.2.5, but in brief is as follows. The prey and bait were co-transformed into CTY10-5d (an *S. cerevisiae* strain, see table 6.8) and grown in liquid culture to midlog phase (OD_{600} 0.2-0.5). The cells were collected and resuspended in an appropriate buffer, at which point they were vortexed in the

presence of chloroform to permeabilise the cells. ONPG was added and they were incubated at 30°C until the reaction turned yellow, at this point the reaction was stopped by adding 1M Na_2CO_3. Typically, samples were incubated for 2-30 mins. Reactions were centrifuged at top speed to remove cell debris and the yellow colour was quantified by spectroscopy (OD_{420}). The OD_{420}, the OD_{600} and volume of culture and reaction time were all used to calculate the β- Gal units.

This assay was done to investigate the interaction between LexABD-Pol31 and the Gal4AD-tagged positives from the two-hybrid screen. The results are plotted in Figure 2.3, below; see Table 8.1, appendix A for raw data.

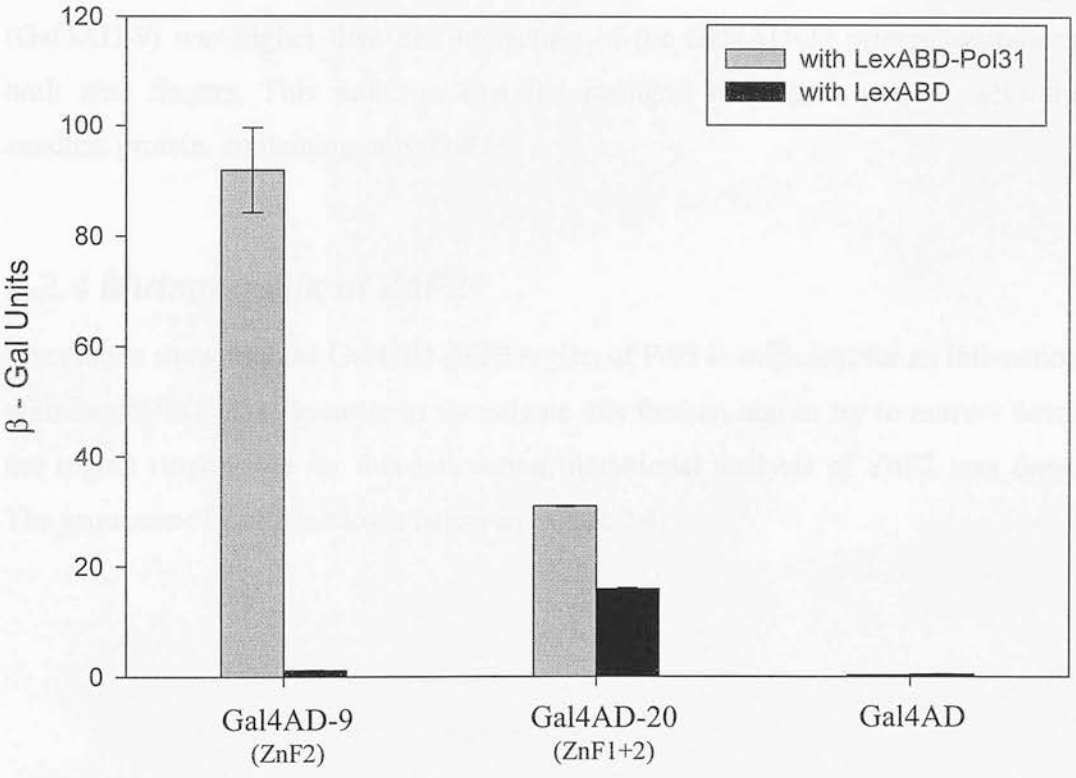

Figure 2.3. β- Gal assay done with the positives from the two-hybrid screen and either LexABD-Pol31 or LexABD. LexABD is expressed from the empty plasmid, pBTM116. Gal4AD is expressed from the empty vector pACT. Assays were done in triplicate. Column height is the mean of the values and error bars are the standard deviation from the mean.

The results show that LexABD-Pol31 is able to interact with the positive interactors as identified from the two-hybrid screen. Furthermore, although there is a surprising level of background with Gal4AD-20 and LexABD, the other controls are behaving as expected, with LexABD and Gal4AD not producing detectable interaction levels. The reason for this higher level of background in Gal4AD-20 could be that the two zinc finger construct is able to interact directly with DNA, therefore Gal4AD-20 comes into contact with the promoter without needing to be bound to the DNA binding domain.

The assay also shows that the β- Gal units of the interaction between LexABD-Pol31 and Gal4AD-9 and between LexABD-Pol31 and Gal4AD-20 are not identical. The interaction with LexABD-Pol31 with the positive containing the single zinc finger (Gal4AD-9) was higher than the interaction of the Gal4AD-20 protein containing both zinc fingers. This indicates that the strongest binding is present with the smallest protein, containing only ZnF2.

2.2.4 Mutagenesis of ZnF2

The results show that the Gal4AD-ZnF2 region of Pol3 is sufficient for an interaction with LexABD-Pol31. In order to investigate this further, and to try to narrow down the region responsible for this interaction, mutational analysis of ZnF2 was done. The sequence of ZnF2 is shown below in Figure 2.4.

Figure 2.4. Sequence alignment of the ZnF2 of the catalytic subunit of polδ. Amino acids in red are those thought to be involved in co-ordinating the metal ion (zinc). Arrow heads indicate the mutations planned. Numbers are amino acid numbers corresponding to the *S. cerevisiae* sequence. The *S. cerevisiae* fragment illustrated corresponds to the smallest clone found in the two-hybrid screen (Gal4AD-9). *Sc*, *S. cerevisiae*; *Sp*, *S. pombe*; *Ca*, *C. albicans*; *Bt*, *B. taurus*; *Mm*, *M. musculus*; *Hs*, *H. sapiens*; *Rn*, *R. norvegicus*; *Ma*, *M. auratus*; *Dm*, *D. melanogaster*.

The metal ion co-ordination in the zinc finger is most likely to be carried out by any four of the amino acids shown in red in Figure 2.4 above (CCHCC). The possibilities for this co-ordination are summarised in Figure 2.5, below.

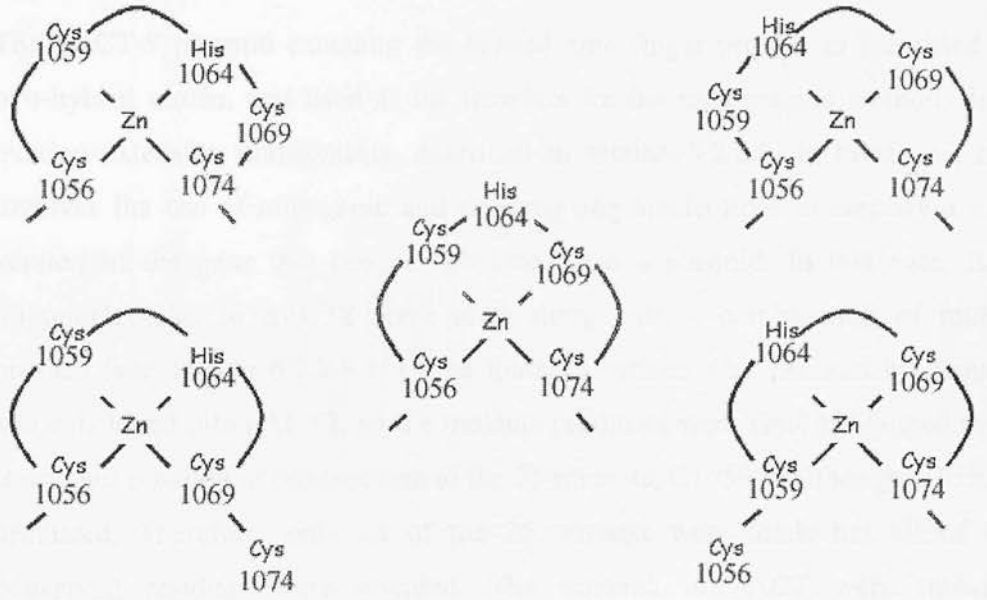

Figure 2.5. Possibilities for metal ion co-ordination in ZnF2 in the catalytic subunit of polδ. See text for further details. Figure courtesy of Dr. S. MacNeill.

As the CCHCC amino acids are probably central to the structure of the zinc finger as shown in Figure 2.5, it was decided to mutate these to alanine. Cysteine is a nucleophilic amino acid, and so it was decided that conservative mutations to serines (also nucleophilic) would also be made at the four cysteines. As can be seen in the sequence alignment in Figure 2.4 above, there are some amino acids that are highly conserved, even though they are probably not involved directly in the metal ion co-ordination in the zinc finger. These amino acids are: E1046, E1047, K1048, R1051, L1052, W1053, T1054, Q1055, Q1057, R1058, N1062, E1066, K1072, F1077-Y1078, and R1080. These amino acids were mutated individually to alanine except for F1077 and Y1078 that were mutated at the same time to make a double mutant.

Another mutant was made in which amino acid K1085 was mutated to a stop codon to investigate a smaller protein. In total there are 21 sites that were mutated to produce a total of 25 mutants. (Mutations E1046A, E1047A, K1048A, L1052A, Q1055A, Q1057A, R1058A, N1062A, K1072A and K1085 to Stop were made by Dr. S. MacNeill.)

The pACT-9 plasmid encoding the second zinc finger protein as identified in the two-hybrid screen, was used as the template for the mutagenesis method, the PCR overlap extension mutagenesis, described in section 6.2.2.6. In brief, this method involves the use of mutagenic and flanking oligonucleotides to amplify a mutated version of the gene that can be subcloned into a plasmid. In this case, flanking oligonucleotides to pACT2 were used along with a combination of mutagenic primers (see section 6.2.2.8.11). The mutated version was checked by sequencing and subcloned into pACT2, so the mutants produced were Gal4AD-tagged proteins. It was not possible to produce one of the 25 mutants, C1059A, although C1059S was produced. Therefore, only 24 of the 25 mutants were made but all of the 21 conserved residues were mutated. The mutants in pACT were transformed individually into the *S. cerevisiae* strain CTY10-5d, along with pBTM116-Pol31, the plasmid encoding LexABD-Pol31. The ability of the Gal4AD-mutants to interact with LexABD-Pol31 was investigated by doing a liquid culture β- Gal assay as before (described fully in section 6.2.2.5). The mutants were compared to wild-type, where binding of wild-type (Gal4AD-9) is 100%. Results are shown in Figure 2.6; see Table 8.2, appendix A for raw data.

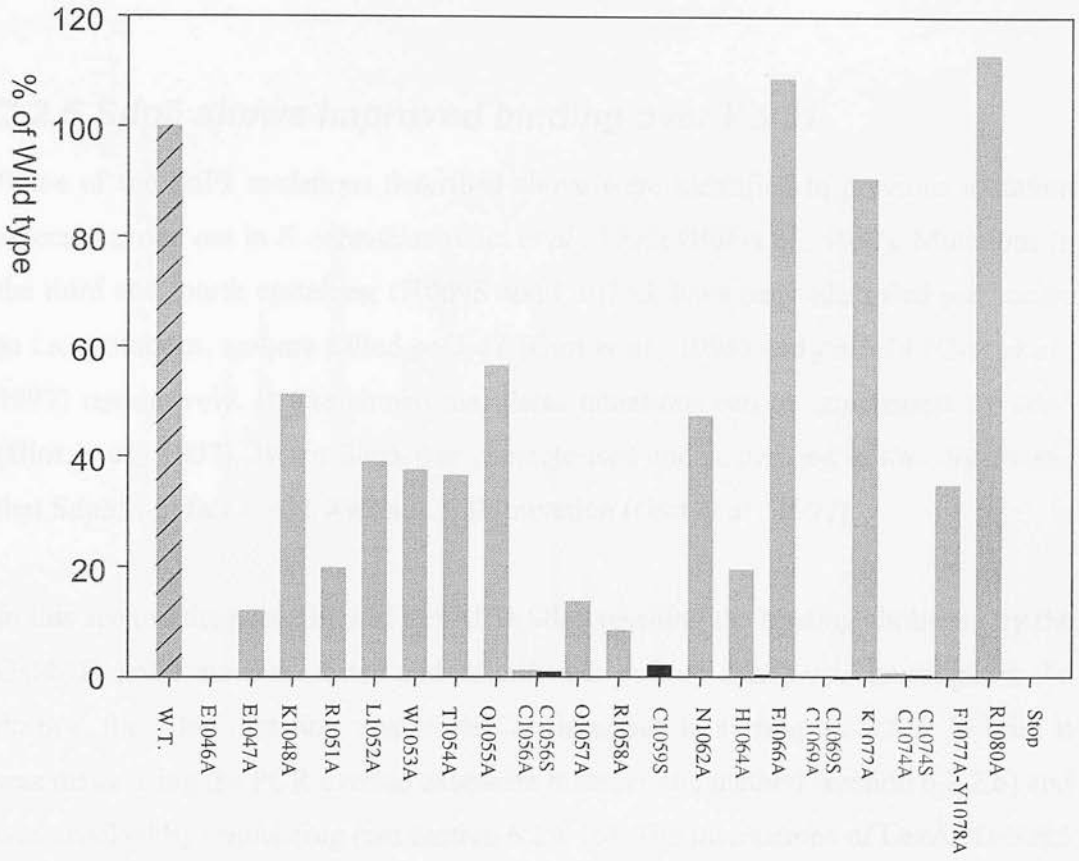

Figure 2.6. Liquid culture β- Gal assay showing the relative strength of the interaction of the Gal4AD-zinc finger mutants when compared to wild type (100%). Wild type is Gal4AD-9. Black columns represent mutations to serine and grey columns represent mutations to alanine. In all cases the assay was done against LexABD-Pol31. "Stop" is amino acid K1085 mutated to a stop codon.

The results show that all of the cysteines appear to be essential for binding as mutating them either to alanine or to serine abolishes binding. The histidine (H1064) appears not to affect binding to a high degree, which suggests that the zinc is tetrahedrally co-ordinated by the four cysteines rather that by some other arrangement involving the histidine (shown in Figure 2.5, in the middle). Mutant E1046A abolishes binding to LexABD-Pol31, although the other point mutations, for example R1058A, whilst showing reduced binding are still able to bind to some extent. Mutations E1066A, K1072A and R1080A are not affected in their ability to bind LexABD-Pol31, they are able to bind to the same extent as wild type. The

mutation in which amino acid K1085 is mutated to a stop codon results in the complete abolition of binding.

2.2.5 Sdp5 shows improved binding over Pol31

Some of the ZnF2 mutations described above were identified in previous mutation screens carried out in *S. cerevisiae* (Giot *et al.,* 1995; Giot *et al.,* 1997). Mutations in the third and fourth cysteines, C1069S and C1074S, have been identified previously as t.s. mutations, and are called *pol3-11* (Giot *et al.,* 1995) and *pol3-13* (Giot *et al.,* 1997) respectively. It was shown that these mutations can be suppressed by Sdp5 (Giot *et al.,* 1997). When Sdp5 was characterised and sequenced it was discovered that Sdp5 is in fact Pol31 with a K358E mutation (Giot *et al.,* 1997).

In this section the possibility of LexABD-Sdp5 rescuing the binding abolished by the Gal4AD-ZnF2 mutants (tested and described in section 2.2.4) was investigated. To do this, the Sdp5 mutation was created as described in section 6.2.2.8.7. In brief it was made using the PCR overlap extension mutagenesis method (section 6.2.2.6) and was checked by sequencing (see section 6.2.1.16). The interactions of LexABD-Sdp5 and all of the Gal4AD-ZnF2 mutants were investigated by doing a β- Gal assay as described previously. Results are shown in Figure 2.7; see Table 8.2, appendix A for raw data.

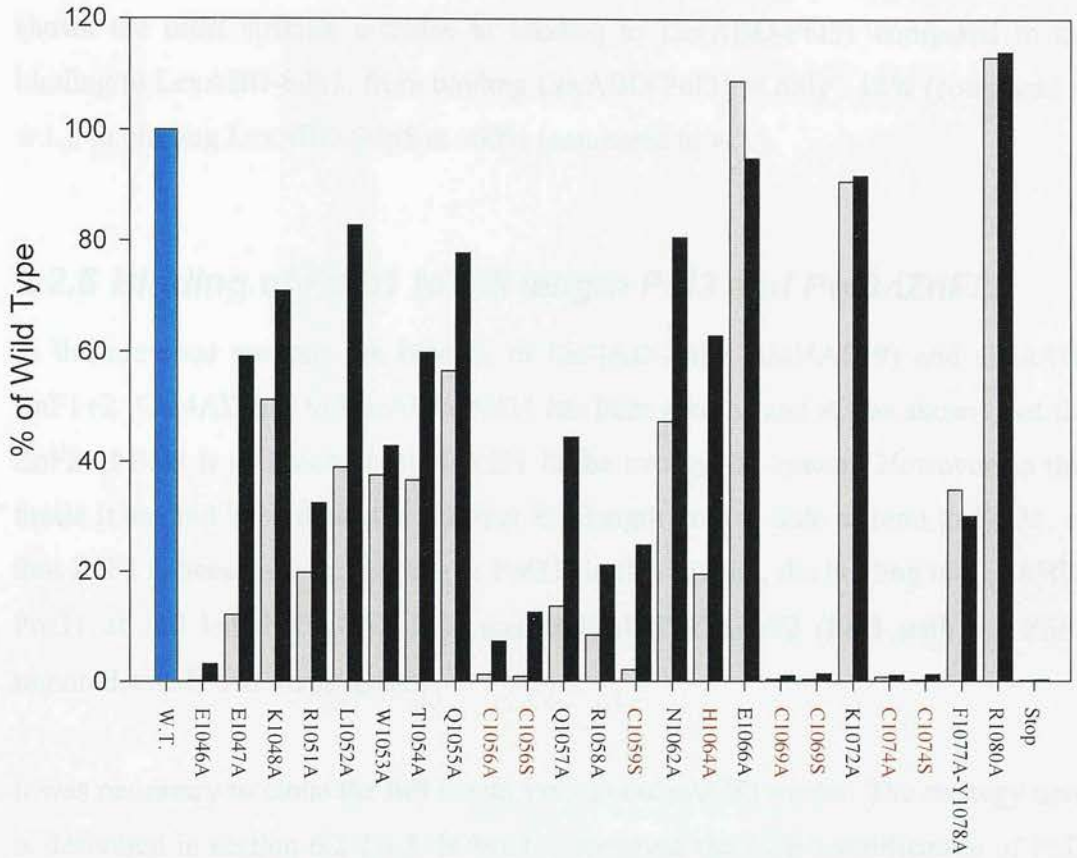

Figure 2.7. Liquid culture β- Gal assay with the Gal4AD-ZnF2 mutants and LexABD-Sdp5 (a mutant of Pol31). Mutants were described in section 2.2.4. The ZnF2 mutants were compared to wild type, Gal4AD-9, where binding of wild type is 100%. "Stop" is amino acid K1085 mutated to a stop codon. Black columns represent assay done with LexABD-Sdp5; grey columns represent assay done with LexABD-Pol31. Blue column is wild type with both LexABD-Pol31 and LexABD-Sdp5.

From the results in Figure 2.7, it can be seen that in general the mutants appear to bind better to LexABD-Sdp5 than to LexABD-Pol31. C1056A, C1056S, C1059S and H1064A show some improvement in binding (about 3 to 4 times) compared to w.t. Interestingly, C1069A, C1069S, C1074A, C1074S do not show any increase in binding to LexABD-Sdp5 when compared to LexABD-Pol31, which suggests that these two cysteines are very important in the binding to LexABD-Pol31. Of the other mutants, E1046A, W1053A, E1066A, K1072A, F1077A-Y1078A, R1080A and the Stop mutation all have similar level of binding to LexABD-Sdp5 and LexABD-Pol31. The mutants E1047A, K1048A, R1051A, L1052A, T1054A, Q1055A,

Q1057A, R1058A, and N1062A show some improvement in the binding to LexABD-Sdp5 when compared to LexABD-Pol31. Mutant E1047A is the one that shows the most striking increase in binding to LexABD-Pol31 compared to the binding to LexABD-Sdp5, from binding LexABD-Pol31 at only ~12% (compared to w.t.), to binding LexABD-Sdp5 at ~60% (compared to w.t.).

2.2.6 Binding of Pol31 to full length Pol3 and Pol3ΔZnF2

In the previous sections the binding of Gal4AD-ZnF2 (Gal4AD-9) and Gal4AD-ZnF1+2 (Gal4AD-20) to LexABD-Pol31 has been studied and it was shown that the ZnF2 of Pol3 is sufficient to bind Pol31 in the two-hybrid system. However, in this thesis it has not been demonstrated that full length Pol3 is able to bind to Pol31, or that ZnF2 is necessary for binding to Pol31. In this section, the binding to LexABD-Pol31 of full length Gal4AD-Pol3 and Gal4AD-Pol3ΔZnF2 (Pol3 with the ZnF2 region deleted) was investigated.

It was necessary to clone the full length Pol3 in the pACT2 vector. The strategy used is described in section 6.2.2.8.5. In brief, it involved the PCR amplification of Pol3 from *S. cerevisiae* genomic DNA, and its subsequent cloning into pACT2. A similar technique was used for cloning Pol3ΔZnF2 into pACT2 (section 6.2.2.8.5). The plasmids pACT-Pol3 and pBTM116-Pol31 were co-transformed into the *S. cerevisiae* strain CTY10-5d. In another transformation, plasmids pACT-Pol3ΔZnF2 and pBTM116-Pol31 were co-transformed into CTY10-5d. Colonies were allowed to grow on appropriate media, and then were grown in liquid culture so a β- Gal assay could be performed as described before and in section 6.2.2.5. The results of the assay are shown in Figure 2.8; see Table 8.3, appendix A for raw data.

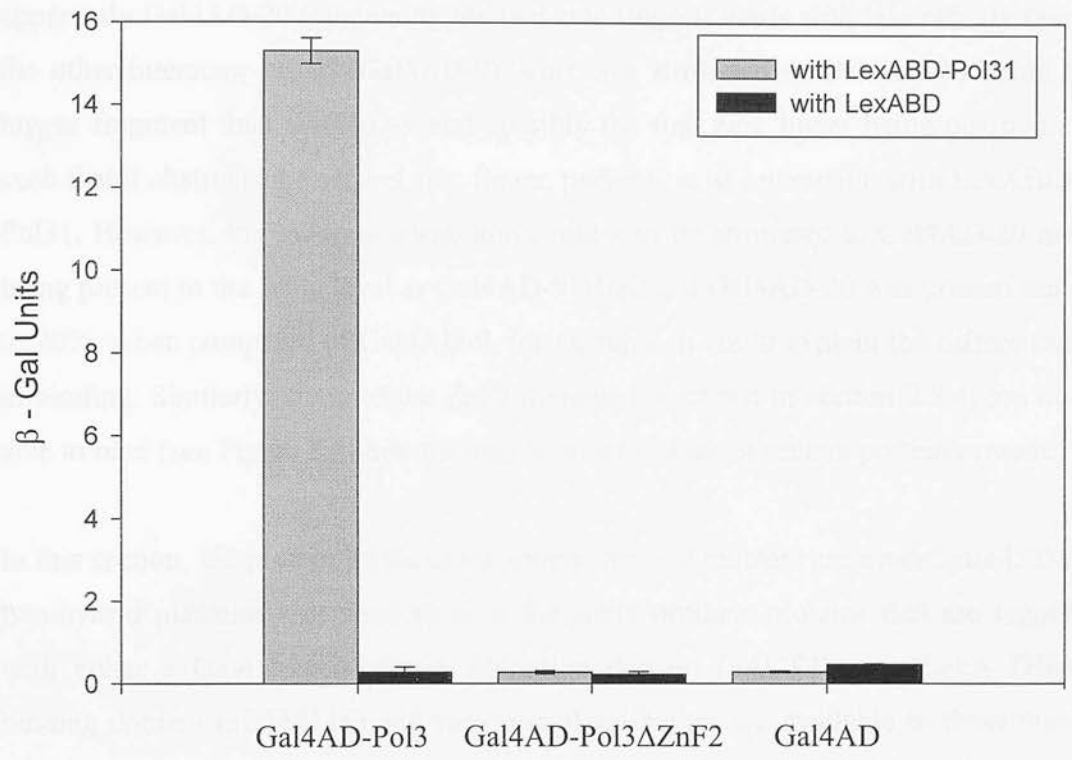

Figure 2.8. Liquid culture β- Gal assay with full length Gal4AD-Pol3 and Gal4AD-Pol3ΔZnF2. Grey columns are the assay done with LexABD-Pol31 and black columns are the assay done with LexABD. Assays were done in triplicate, column height is the mean of the values and error bars are the standard deviation from the mean.

As can be seen from Figure 2.8 full length Gal4AD-Pol3 is able to bind to LexABD-Pol31 as expected and it appears to be binding with a similar strength to that of Gal4AD-20 (30 β- Gal units), which contains both zinc fingers. Gal4AD-Pol3ΔZnF2 is, on the other hand, not able to bind to LexABD-Pol31. This indicates that the ZnF2 is necessary for the binding of Pol3 to Pol31.

2.2.7 Protein levels of two-hybrid proteins

In the previous sections, much has been said about the relative level of binding of both the Gal4AD-tagged Pol3 interactors found in the two-hybrid screen and the Gal4AD-ZnF2 mutants that were made (see sections 2.2.3.2, 2.2.4 and 2.2.5). However, the difference in the levels of binding may be due to differences in the

protein levels of different interactors or mutants. In Figure 2.3 it is shown that apparently Gal4AD-20 (containing the two zinc fingers) binds with less affinity than the other interactor tested (Gal4AD-9). This was attributed to Gal4AD-20 being a bigger fragment than Gal4AD-9 and possibly the first zinc finger being positioned such that it obstructs the second zinc finger, preventing its interaction with LexABD-Pol31. However, the reduced interaction could also be attributed to Gal4AD-20 not being present to the same level as Gal4AD-9. Hence, if Gal4AD-20 was present only to 20%, when compared to Gal4AD-9, for example, it could explain the differences in binding. Similarly, some of the ZnF2 mutants (described in section 2.2.4) are not able to bind (see Figure 2.6) but this may be due to a lack of mutant protein present.

In this section, the protein levels of the interactors and mutants are investigated. The two-hybrid plasmids that were used in the study produce proteins that are tagged with either a Gal4 transcriptional activation domain (pACT2) or a LexA DNA binding domain (pBTM116) and commercial antibodies are available to these tags. Therefore, Western analysis was performed to elucidate the protein levels of the proteins used in the two-hybrid assays.

2.2.7.1 Protein levels of the interactors from the two-hybrid screen

The protein levels of the proteins that were identified as binding to LexABD-Pol31 in the two-hybrid screen (see section 2.2.2) were investigated using a Gal4 antibody. Gal4AD-7, -9, -10, -11, -15, -20 and the empty pACT, which produces Gal4AD alone, were transformed separately into the *S. cerevisiae* strain CTY10-5d, and total protein was extracted (see section 6.2.1.22). To ensure that equal amounts of protein were loaded, a Bio-Rad Protein Assay kit (see section 6.2.1.23) was used to measure the total protein concentration. For the Western blot, 10 μg of total protein was loaded. The Western blot was done as described in section 6.2.1.25, results are shown in Figure 2.9, below.

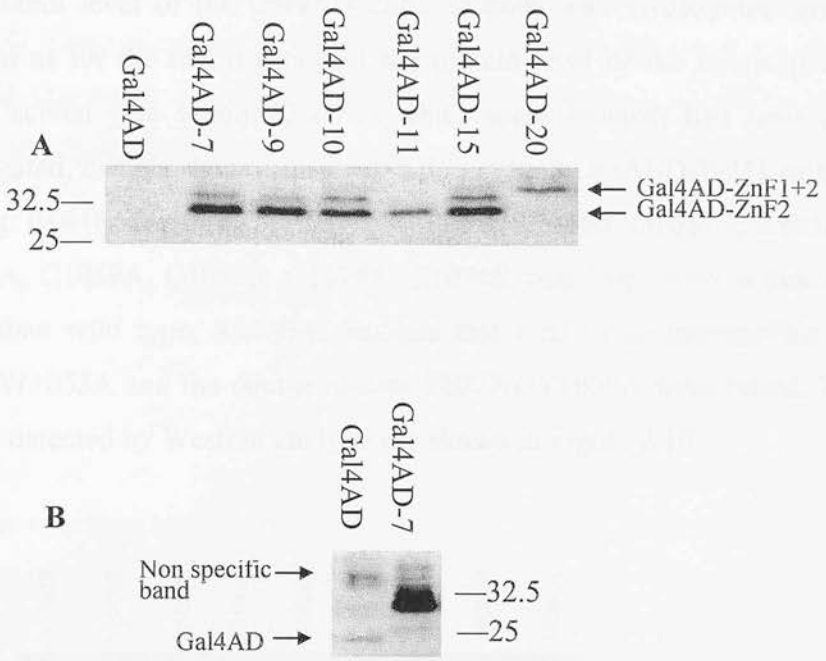

Figure 2.9. A, Western blot of the interactors of the two-hybrid system. B, longer exposure of a fragment of the same western to show the presence of Gal4AD in the "Gal4AD" lane. 10 μg of total protein was loaded in each lane as estimated by a protein assay kit. Numbers to the left of the blot are protein sizes in kDa.

As can be seen from Figure 2.9 the protein level appears to be approximately the same. The exception is the empty vector, pACT, which appears to produce Gal4AD to a very reduced level, so reduced that it was necessary to expose the film for an extended period (an extra 14.5 min) to be able to see the protein (see Figure 2.9, B). Gal4AD-20 appears to be present to a lower level than the other interactors, which could account for its reduced level of binding with LexABD-Pol31, when compared to Gal4AD-9. It is not known what the extra bands present above the expected Gal4AD-ZnF2 band are, although it is tempting to speculate that they are indicative of phosphorylation, or a similar modification occuring in the ZnF2.

2.2.7.2 Protein levels of ZnF2 mutants

The protein level of the Gal4AD-ZnF2 mutants was investigated using the same protocol as for the investigation of the protein level of the interactors of the two-hybrid screen (see section 2.2.7.1). Only some mutants had their protein level investigated, namely those mutants that do not bind LexABD-Pol31 or show a <20% binding: E1046A, E1047A, R1051A, C1056A, C1056S, Q1057A, R1058A, C1059S, H1064A, C1069A, C1069S, C1074A, C1074S, and Stop. Also, a mutant that binds better than wild type: R1080A; mutants that bind to an intermediate degree (30-50%): W1053A and the double mutant F1077A-Y1078A were tested. The levels of protein detected by Western analysis are shown in Figure 2.10.

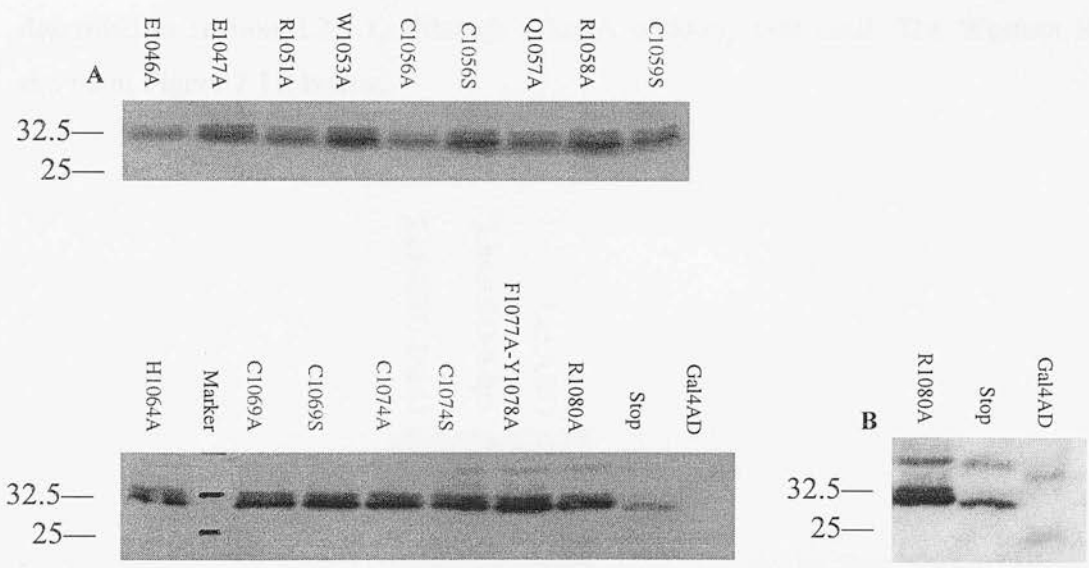

Figure 2.10. A, Western blot of some of the Gal4AD-ZnF2 mutant proteins. B, longer exposure of the same western to show the presence of protein in the "Stop" lane and in the Gal4AD lane. 10 μg of total protein was loaded in each lane as estimated by a protein assay kit. Numbers to the left of the blot are protein sizes in kDa.

As can be seen in Figure 2.10 there appears to be some variation between the level of protein present. The mutant that was able to bind better than wild type (R1080A) produced the same amount of detectable protein as the mutants that are only able to bind at 30-50% of wild type (W1053A and F1077A-Y1078A). This level of protein

was the same as most of the mutants that are not able to bind or showed <20% binding (E1047A, C1056S, R1058A, H1064A, C1069A, C1069S, C1074A and C1074S). Some of the mutants that are not able to bind show a slight reduction in the level of detectable protein (E1046A, R1051A, C1056A, Q1057A, and C1052S). "Stop" and Gal4AD show a marked reduction in the level of detectable protein.

2.2.7.3 Bait protein levels

The level of binding of Gal4AD-ZnF2 mutants differs if LexABD-Sdp5 is used instead of LexABD-Pol31 (see section 2.2.5). The difference could be explained if LexABD-Pol31 was present at a different level than LexABD-Sdp5. Therefore, a Western was done to investigate the protein levels. The protocol was the same as that described in section 2.2.7.1, although a LexA antibody was used. The Western is shown in Figure 2.11, below.

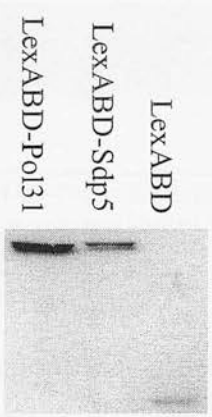

Figure 2.11. Western blot of LexABD-Pol31, LexABD-Sdp-5 and LexABD. 10 μg of total protein was loaded in each lane as estimated by a protein assay kit.

As can be seen in Figure 2.11, the protein level is high in LexABD-Pol31, when compared to LexABD-Sdp5 and the protein expressed by the empty vector (LexABD) has the lowest level.

2.2.7.4 Protein levels of Pol3 fragments

As can be seen from section 2.2.6, LexABD-Pol31 is able to bind to full length Gal4AD-Pol3 but not to Gal4AD-Pol3ΔZnF2. This is presumably because the second zinc finger is necessary for the binding to LexABD-Pol31 in the two-hybrid system. In order to establish if this is definitely the case, the protein levels of the two Gal4AD-Pol3 proteins were investigated. For this, a Western blot of Gal4AD-Pol3 and Gal4AD-Pol3ΔZnF2 was carried out. As in the previous Western blots the same amount (10 μg) of total protein was loaded in each lane. Figure 2.12 below shows the result of the Western, using the anti-Gal4 antibody.

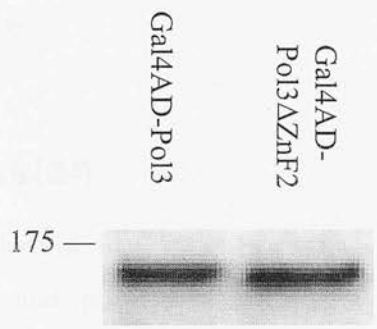

Figure 2.12. Western blot of Gal4AD-Pol3 and Gal4AD-Pol3ΔZnF2. Samples were run on an 8% polyacrylamide gel. 10 μg of total protein was loaded in each lane as estimated by a protein assay kit. The number to the left of the blot is protein size in kDa.

From Figure 2.12 above it appears that both Gal4AD-Pol3 and Gal4AD-Pol3ΔZnF2 are present at comparable levels.

2.2.8 Structure of ZnF2

Structural information on the ZnF2 region of Pol3 will be very interesting in order to comprehend the region more fully; unfortunately the structure of neither Pol3 nor ZnF2 have been solved. Since the ZnF2 region of Pol3 has no known structural homologues it is not possible to compare ZnF2 with a previously solved structure. Therefore the web based PredictProtein structure prediction programme (http://www.embl-heidelberg.de/predictprotein/predictprotein.html) was used. The

sequence submitted to the server was the ZnF2 region and some adjacent sequence. Figure 2.13, shows the protein sequence and the PredictProtein results.

```
KALYDVRDLE EKYSRLWTQC QRCAGNLHSE VLCSNKNCDI FYMRVKVKKE LQEKVEQLSK W
LLLHHHHHHH HHHHHHHHHH HHHHLLLLLE EEEELLLLLE EEEEEEEHHH HHHHHHHHHH L
9221178888 8888888886 6532222530 2420456533 4555320266 8889987510 8
```

Figure 2.13. Structure prediction of the ZnF2 according to the PredictProtein structure prediction programme. H=helix, E=extended (sheet), L=loop. Numbers indicate reliability index for prediction (0=low, 9=high).

This suggestion for a structure of ther ZnF2 region will de discussed more fully in section 2.3, below.

2.3 Discussion

When the two-hybrid work was planned it was hoped that interactions between LexABD-Pol31 and previously unidentified proteins would be found. Instead, Gal4AD-Pol3, a protein previously known to interact with Pol31 was identified. What made this interesting was the fact that it was not the whole of Pol3 that was found to interact with LexABD-Pol31, but the C- terminal region. Currently, there is very limited functional information for this region: it contains two putative zinc fingers and mutations in this region can be rescued by Sdp5 (a Pol31 mutant, see section 2.2.5; (Giot *et al.*, 1995; Giot *et al.*, 1997)). Two independent Pol3 clones were obtained (as would be expected for a "real" interaction) and the smallest was formed almost exclusively of the second zinc finger. Confirmation of the interactions showed that this clone, was the strongest interactor. The other clone, that corresponding to both C- terminal zinc fingers, has a weaker interaction and the largest level of background. This suggests that the second zinc finger is the region involved in the binding and that the interactor containing both zinc fingers contains a large amount of protein that is not involved in the binding and actually hinders it. The reason for the large level of background observed with the largest clone could be due to the two zinc fingers being able to bind directly to DNA, which would bring

the transcriptional activation domain close to the promoter region where it would be able to activate the reporter gene.

In order to investigate if the difference in the interaction of Gal4AD-20 and Gal4AD-9 with LexABD-Pol31, was due to a difference in the protein level of the interactors a Western blot was performed on the interactors. The results can be seen in Figure 2.9. It appears that Gal4AD-20 did have a slightly reduced level of protein when compared to Gal4AD-9, which could account for its reduced level of binding. It is hard to quantify subtle differences in protein levels in a Western blot. In the β- Gal assay Gal4AD-20 binding to LexABD-Pol31 is around 20% of Gal4AD-9 binding. Since the protein level of Gal4AD-20 is probably higher than 20% of Gal4AD-9 it can be deduced that the protein level is not wholly responsible for the reduced binding observed with Gal4AD-20.

In the mutational analysis of the second zinc finger, it was found that mutations of any of the four cysteines of ZnF2 results in the abolition of binding, whereas the histidine mutant is still able to bind. The fact that the H1064A mutation was still able to bind to LexABD-Pol31, suggests that this residue is not involved in co-ordinating the zinc ion. Therefore, it is likely that the four cysteines are the structurally important residues of the second zinc finger, co-ordinating the zinc ion. This likely pattern of zinc co-ordination is shown in the centre of Figure 2.5.

Mutant E1046A also abolishes binding completely, which suggests that this amino acid is important either structurally or directly for binding. The mutation in which amino acid K1085 was mutated to a stop codon was unable to bind to LexABD-Pol31. This suggests that either the amino acids from 1085 to 1097 are crucial for binding or that a deletion of these amino acids causes the protein not to be expressed or perhaps to fold incorrectly or to be degraded. Some mutants are able to bind although they do so at a reduced level (<20%), these mutants (E1047A, R1051A, Q1057A, and R1058A) are thought to be involved in maintaining the structure of ZnF2.

The protein levels of the mutants were investigated to ensure that the mutants that are not able to bind still have protein present and at a comparable level to those that do. The results (shown in Figure 2.10) show that the mutant "Stop" has a marked reduction in the protein level present, which could be responsible for it not being able to bind to LexABD-Pol31. The rest of the mutants appear to vary with respect to protein levels. However, it was observed that mutants that bind better than wild type, those that are able to bind only to <20% of wild type levels and those mutants that are able to bind to 30-50% of wild type levels have comparable amounts of protein present. Hence, it is probably reasonable to assume that the small difference in the protein levels of the point mutants is not the cause of the differences in the ability of the mutants to bind to LexABD-Pol31. The differences in binding are probably due to an effect of the mutation on the structure of the ZnF2 or due to a direct involvement in binding to LexABD-Pol31.

Interestingly, when the Gal4AD-ZnF2 mutants were investigated in terms of their ability to bind to LexABD-Sdp5 (a LexABD-tagged point mutation of Pol31), it was discovered that most of the Gal4AD-ZnF2 mutants were able to bind to LexABD-Sdp5 better than they were able to bind to LexABD-Pol31. The most striking increase in binding is that of E1047A, that changes from ~12% of binding to LexABD-Pol31 (in comparison to w.t.) to almost 60% of binding to LexABD-Sdp5 (in comparison to w.t.) The other mutants that show an increase in binding to LexABD-Sdp5 when compared to LexABD-Pol31 typically show an increase of more than 10%. Mutants E1047A, R1051A, Q1057A, R1058A, and H1064A appear to be interesting as their level of binding with LexABD-Pol31 was less than 20% (in comparison to w.t.) which is indicative of weak binding, however, their binding with LexABD-Sdp5 is >20%. LexABD-Sdp5 has had a strong effect on these mutants. On the other hand the binding of some mutants appears not to be affected by the difference between LexABD-Sdp5 and LexABD-Pol31. This is the case for E1066A, K1072A, R1080A and "Stop", which have a very similar level of binding with LexABD-Pol31 and LexABD-Sdp5. Interestingly they are all grouped near the C-terminus of ZnF2, indicating that maybe this region is not affected by the Sdp5

mutation. Hence, probably the region of Pol31 mutated in Sdp5 is involved in the binding to the region upstream of E1066A of ZnF2.

The four cysteines that are thought to be involved in the binding to zinc behaved differently when their binding with LexABD-Sdp5 was investigated. (The cysteine mutations to alanine and serine behaved similarly and thus will be referred to simply as cysteine mutations). C1056 and C1059 showed a three or four times increase in binding to LexABD-Sdp5 compared to LexABD-Pol31. However, the binding of C1069 and C1074 to LexABD-Sdp5 was very similar to the binding to LexABD-Pol31, i.e. there was no binding. This difference in binding between the two pairs of cysteines is interesting as it suggests that the four amino acids involved in the binding to zinc act differently. A possibility could also be that LexABD-Sdp5 does not affect mutants located closer to the C- terminus than C1069, as those mutants appear to have the same level of binding to LexABD-Pol31 and LexABD-Sdp5 (see above). Interestingly however, the screen that identified Sdp5 identified it as a mutant that is able to rescue the t.s. phenotype of two C to S mutants in ZnF2 (C1069S and C1074S; (Giot *et al.,* 1995; Giot *et al.,* 1997)). Hence, it appears, in that work, that Sdp5 does affect the mutants located to the C- terminus of C1069. Even though in the previous work the t.s. phenotype was suppressed, in the work described in this chapter it was found that the binding to LexABD-Sdp5 is still deficient. The cells were grown at 30°C, so it is conceivable that a difference might be found if the cells were to be grown at a different temperature.

The difference between the binding of the mutants to LexABD-Pol31 and LexABD-Sdp5 could also be accounted for by LexABD-Sdp5 having a different protein level than LexABD-Pol31. From the results of a Western blot (see Figure 2.11) it appears that there is slightly more LexABD-Pol31 present than LexABD-Sdp5. Hence, given that the overall effect of LexABD-Sdp5 increases the binding of the ZnF2 mutants, compared to LexABD-Pol31 it is most likely that this effect is not related to the difference in protein levels. It is most likely that the apparent increased binding observed with LexABD-Sdp5, when compared with LexABD-Pol31, is the result of the K358E mutation, a change from a positive amino acid to a negative one. It is

likely that the region around amino acid 358 of Pol31 is involved in the binding to Pol3, via the ZnF2 region.

The structure prediction of ZnF2 and its mutational analysis gives suggestions for its structure. Most likely it is the four cysteines of the zinc finger which co-ordinate the binding to the zinc, rather than the histidine (although it may affect the overall structure to a small degree). The amino acids in between the second and third cysteines are probably not directly involved in their structure but may have a small effect. From the structure prediction there is a helix region that contains the two first cysteines whereas the next two are in between an extended region and a loop, that precede another helix region. The residues E1046, E1047 and R1051 are probably also involved in the maintaining of the structure.

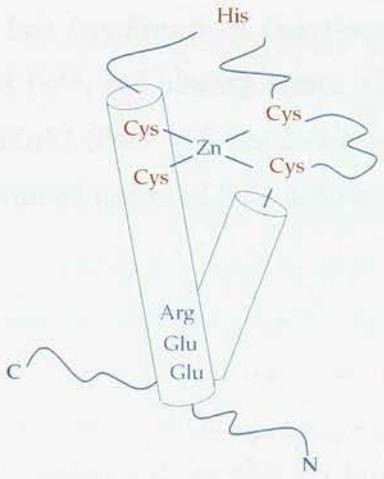

Figure 2.14. Prediction of ZnF2 structure. Amino acids pictured (except for His) are those thought to be important for the structure. Figure courtesy of Dr. S. MacNeill.

The results previously mentioned appear to indicate that Gal4AD-ZnF2 of Pol3 is sufficient for binding to LexABD-Pol31. In order to investigate if the Gal4AD-ZnF2 is not only sufficient but necessary for this binding, a two-hybrid assay was done with full length Gal4AD-Pol3 and Gal4AD-Pol3ΔZnF2. This assay showed that full length Gal4AD-Pol3 was able to bind to LexABD-Pol31. The level of binding was

reduced when compared to Gal4AD-ZnF2 alone (Gal4AD-Pol3: ~15 β- Gal units; Gal4AD-ZnF2(9): ~90 β- Gal units) but it was less different when compared to the clone corresponding to both zinc fingers (Gal4AD-20: ~30 units). When the assay was done on Gal4AD-Pol3ΔZnF2 it was shown that this clone was not able to bind to LexABD-Pol31. The protein levels were investigated to ensure that Gal4AD-Pol3ΔZnF2 is present to a similar level to full length Gal4AD-Pol3. The Western blot (see Figure 2.12) shows that Gal4AD-Pol3 and Gal4AD-Pol3ΔZnF2 are present at what appears to be the same level. Therefore, the putative second zinc finger of Pol3 is necessary for binding to Pol31.

In summary, in this chapter, the two-hybrid system has been used to show that a point mutation in LexABD-Pol31 causes the binding to Gal4AD-ZnF2 of Pol3 to increase, suggesting that this region of Pol31 is required for the interaction of Pol31 with the ZnF2 region of Pol3. Further, using evidence from a two-hybrid screen with LexABD-Pol31 as bait (confirmed by direct mating and liquid assays), mutational analysis of ZnF2 of Pol3, and binding assays with Gal4AD-Pol3 (full length Pol3) and Gal4AD-Pol3ΔZnF2 (Pol3 with ZnF2 deleted), it has been shown that the ZnF2 located in the C- terminal region of Pol3 is both necessary and sufficient for binding Pol31.

3 Fission yeast Pol3-Cdc1 Interactions

3.1 Introduction

The catalytic subunit of fission yeast polδ, Pol3, is known to interact with the B subunit, Cdc1, however, the precise regions involved in this binding are not known. There are several lines of evidence that support the idea that the C- terminus of the catalytic subunit is involved in the direct binding to the B subunit. In *S. cerevisiae* polδ, t.s. mutations that lie in the C- terminus of the catalytic subunit (Pol3), more specifically in the second zinc finger region, can be rescued by a mutation in the B subunit (Pol31; (Giot *et al.,* 1997)), suggesting their possible interaction. This B subunit mutation (Sdp5-15) is a single amino acid change from a lysine (K358) to a glutamic acid (E358), i.e. from a basic amino acid to an acidic one. In *S. cerevisiae* polε, the B subunit has been shown to interact with the catalytic subunit (Dua *et al.,* 2000). Furthermore, if the C- terminus of the polε catalytic subunit is deleted, the binding to the B subunit is abolished. Similarly, in mouse polα, the C- terminus of the catalytic subunit has been shown to interact with the B subunit (Mizuno *et al.,* 1999). In *S. pombe*, it has been shown that the N- terminal 211 amino acids of Pol3 are not required for the binding to Cdc1 (MacNeill *et al.,* 1996). However, the most concrete piece of evidence for the importance of the C- terminus of Pol3 for binding to the B subunit comes from the two-hybrid and mutational analysis work presented in the previous chapter in which it was shown that the ZnF2 region of Pol3 in *S. cerevisiae* is both necessary and sufficient for the binding to Pol31.

As mentioned above, the binding studies of the *S. pombe* proteins are limited to showing that Pol3 lacking the N- terminal 211 amino acids is sufficient to bind to Cdc1 (MacNeill *et al.,* 1996). Therefore, the Pol3-Cdc1 interaction was investigated in more detail in *S. pombe*, with the aim of determining the fine detail of the Pol3 region needed to bind to Cdc1.

3.2 Results

3.2.1 Pol3 can bind Cdc1 in the presence of Cdc27

Firstly, it was decided that confirmation of the binding of Pol3 to Cdc1 would be attempted before narrowing down the region required for binding. The chosen assay system is the β- galactosidase activity assay system (β- Gal assay). In this assay, the proteins to be investigated are tagged with either a Gal4 activation domain fusion (Gal4AD) or a LexA binding domain fusion (LexABD). The plasmids that express the fusion proteins were co-transformed into the *S. cerevisiae* strain CTY10-5d. In this case plasmids pGAD-Pol3, which will produce Pol3 protein tagged with the Gal4AD, and pBTM116-Cdc1, which will produce Cdc1 protein tagged with the LexABD were used. Colonies were obtained by growing in the appropriate medium, they were grown in liquid and a β- Gal assay was performed as described in section 6.2.2.5. Results are shown in Figure 3.1, column 1 below; see Table 8.4, appendix 3 for raw data.

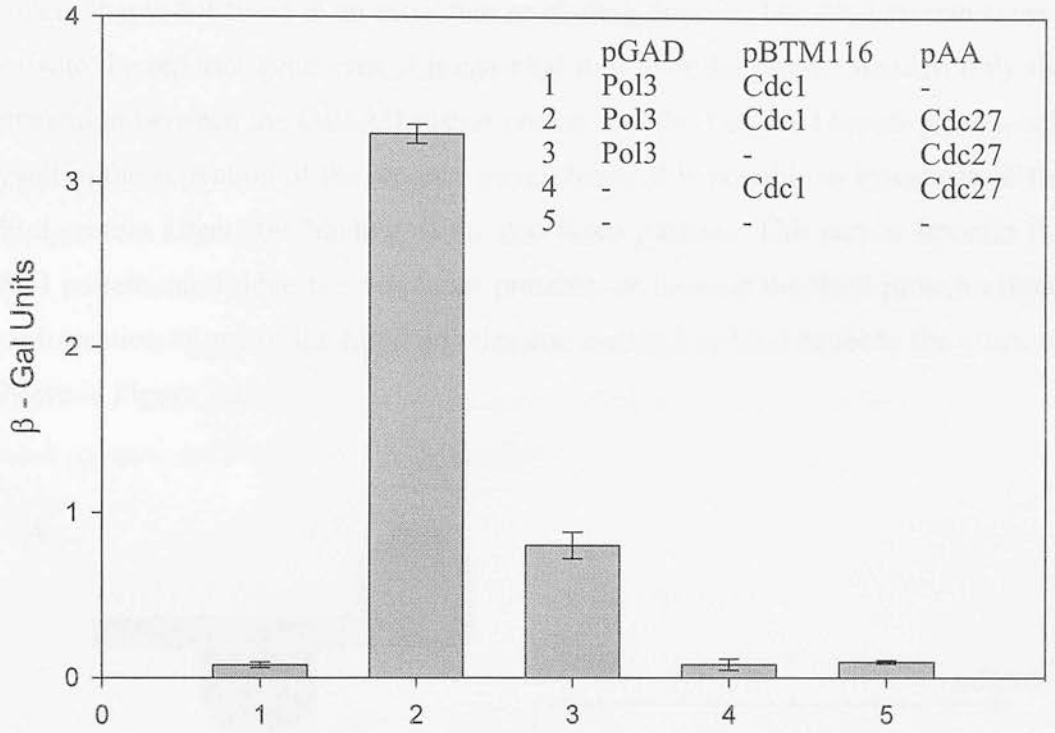

Figure 3.1. Liquid culture β- Gal assay to investigate the binding of Gal4AD-Pol3 to Cdc1. "–" indicates that the empty vector was used. Experiments were done in triplicate, column height is the mean of the values and error bars are the standard deviation from the mean.

The results above show that fission yeast Gal4AD-Pol3 is not able to bind to LexABD-Cdc1 alone in a two-hybrid assay. This is surprising, as *in vitro* studies have shown that Pol3 with the first 211 amino acids deleted is able to bind to Cdc1. Also, work in the previous chapter suggests that Gal4AD-Pol3 and LexABD-Pol31 on their own are able to interact. However, since the two-hybrid in the previous chapter was performed with *S. cerevisiae* proteins in *S. cerevisiae*, the endogenous *S. cerevisiae* C subunit (Pol32) is also present. Hence, the presence of this C subunit might be stabilising the Gal4AD-Pol3/LexABD-Pol31 interaction. Therefore, it was possible that if the C subunit of *S. pombe* polδ (Cdc27) were present then Gal4AD-Pol3 and LexABD-Cdc1 will be able to interact.

This was tested by doing a "three hybrid" assay (see Figure 3.2 for a schematic representation of a "three hybrid" assay). In a "three hybrid" assay, three proteins are

produced, one fused to the Gal4AD, another one fused to the LexABD and a third protein that is not fused to an activation or binding domain. The third protein cannot activate the reporter gene even if it can bind to any of the protein fusions, only the interaction between the Gal4AD fusion protein and the LexABD fusion protein will result in the activation of the reporter gene. Hence, it is possible to investigate if the third protein affects the binding of the two fused proteins. This can be because the third protein can bridge the two fused proteins, or because the third protein affects conformation of one of the fused proteins and causes it to bind better to the other, as shown in Figure 3.2.

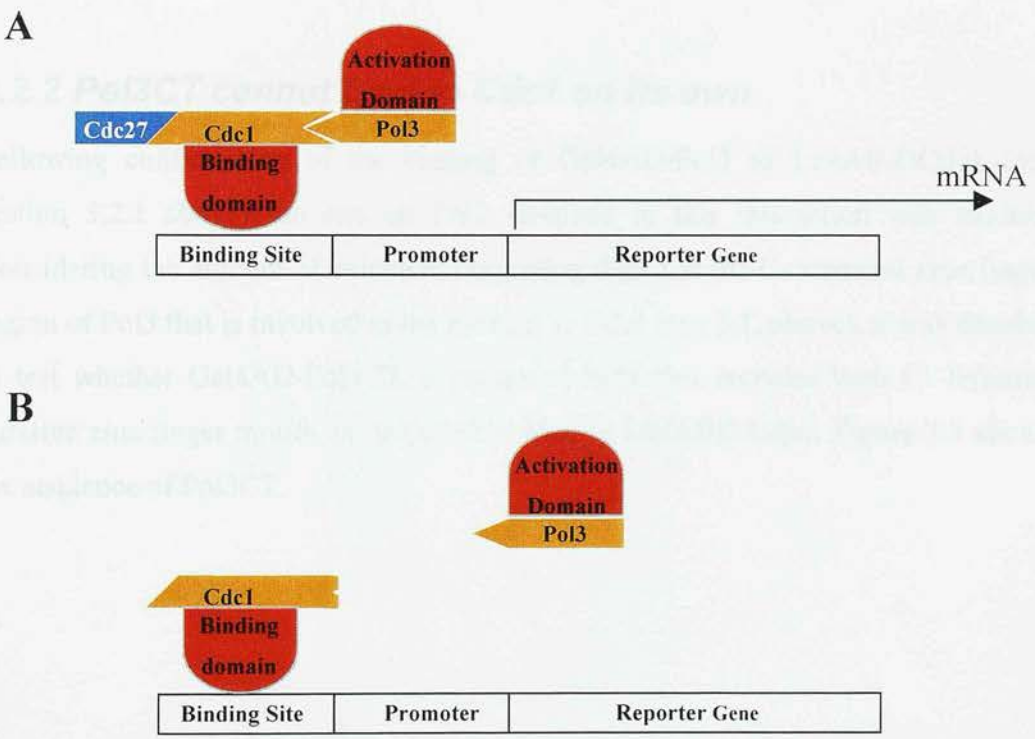

Figure 3.2. Schematic representation of a "three hybrid" assay. **A** The third protein (Cdc27, in blue) binds to the bait protein (LexABD-Cdc1) and causes it to bind to the prey protein (Gal4AD-Pol3). **B** The bait and prey proteins are unable to interact in the absence of the third subuit (in blue) and the reporter gene is not activated.

The "three hybrid" assay was performed by co-transforming pGAD-Pol3, pBTM116-Cdc1 and pAA-Cdc27 into CTY10-5d. Colonies were obtained by growing in the appropriate medium, they were grown in liquid and a β- Gal assay was performed as

described in section 6.2.2.5. As the Cdc27 protein was expressed from the pAA plasmid, which will produce Cdc27 with a myc tag, it means that it will be unable to cause activation of the reporter gene, since it is not fused to the activation or the binding domain. The results of the "three hybrid" assay with Gal4AD-Pol3, LexABD-Cdc1 and myc-tagged Cdc27 are shown in Figure 3.1, columns 2-5. It appears that myc-tagged Cdc27 has a stabilising effect on the binding of Gal4AD-Pol3 with LexABD-Cdc1. The binding is not very strong as only 3 β- Gal units were obtained with Gal4AD-Pol3, LexABD-Cdc1 and myc-tagged Cdc27. However, the negative controls, columns 3, 4 and 5 have values below 1, which indicates that all three proteins are needed for the interaction.

3.2.2 Pol3CT cannot bind to Cdc1 on its own

Following confirmation of the binding of Gal4AD-Pol3 to LexAB-DCdc1 (see section 3.2.1 above) the site on Pol3 involved in this interaction was studied. Considering the amount of evidence suggesting that it is the C- terminal zinc finger region of Pol3 that is involved in the binding to Cdc1 (see 3.1, above), it was decided to test whether Gal4AD-Pol3CT, a region of Pol3 that includes both C- terminal putative zinc finger motifs, is sufficient to bind to LexABD-Cdc1. Figure 3.3 shows the sequence of Pol3CT.

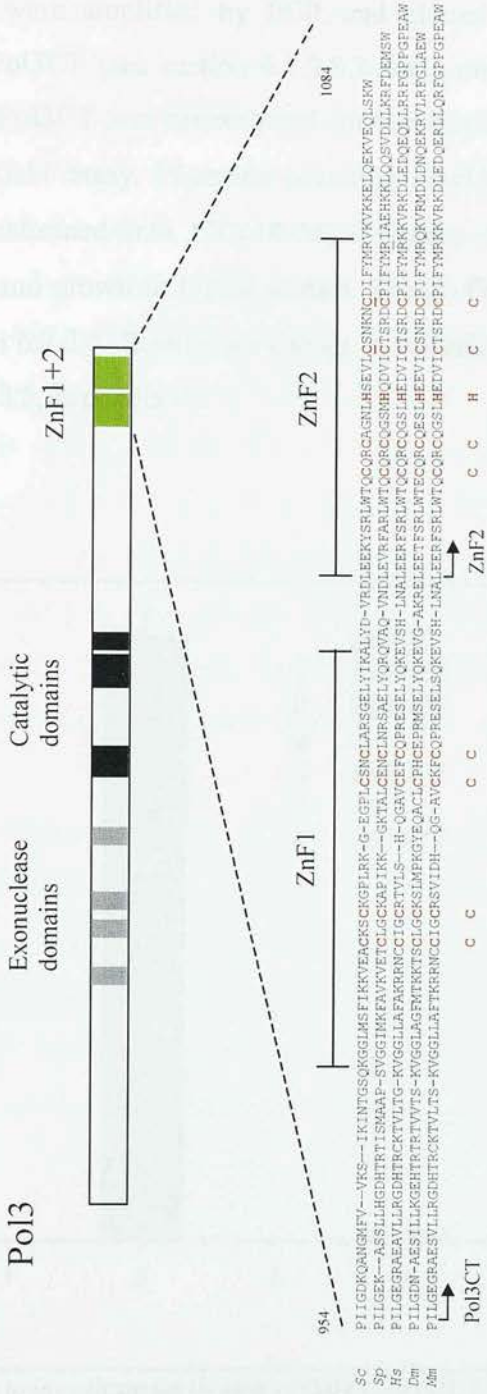

Figure 3.3. Sequence alignment of the C- terminus two zinc fingers of Pol3. Shown in the alignment are the following constructs: Pol3CT, an *S. pombe* clone containing ZnF1+2; ZnF2, a *S. pombe* clone containing ZnF2 only. Amino acids in red are those thought to be involved in the co-ordinating of the zinc in the zinc finger. Amino acids underlined show the location of t.s. mutants. Numbers are amino acid numbers with *S. pombe* numbering. *Sc, S. cerevisiae; Sp, S. pombe; Hs, H. sapiens; Dm, D. melanogaster; Mm, M. musculus.*

It was necessary to create the Gal4AD-Pol3CT protein fusion. For this, sequences encoding Pol3CT were amplified by PCR and cloned into pACT2, which will produce Gal4AD-Pol3CT (see section 6.2.2.8.3 for a more detailed account of the cloning). Gal4AD-Pol3CT was investigated for the binding to LexABD-Cdc1 by a liquid culture β- Gal4 assay. Plasmids encoding Gal4AD-Pol3CT and LexABD-Cdc1 were co-transformed into CTY10-5d. Colonies were allowed to grow on appropriate media and grown in liquid culture. The β- Gal assay was performed as described in section 6.2.2.5. Results are shown in column 1 of Figure 3.4 below; for raw data see Table 8.5, appendix A.

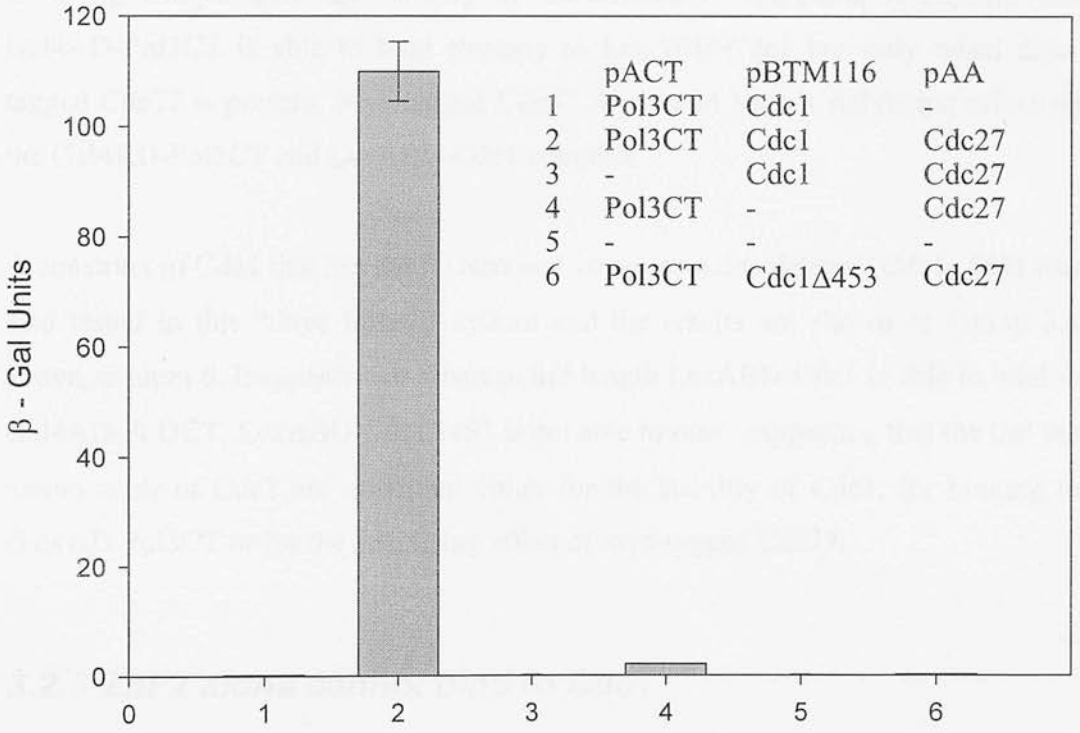

Figure 3.4. β- Gal assay to investigate the binding of Gal4AD-Pol3CT to LexABD-Cdc1. "-" indicates that the empty vector was used. Experiments were done in triplicate, column height is the mean of the values and error bars are the standard deviation from the mean.

It appears from column 1 that Gal4AD-Pol3CT and LexABD-Cdc1 alone do not interact in the two-hybrid system. This was surprising as in *S. cerevisiae* the clone containing the two zinc finger motifs of Pol3, tagged to the Gal4AD is able to

interact with LexABD-Pol31. However, as mentioned in the previous section, it is possible that Pol32 could have had a stabilising effect on the Gal4AD-Pol3/LexABD-Pol31 interactions, and a similar stabilising effect of myc-tagged Cdc27 on Gal4AD-Pol3/LexABD-Cdc1 binding was previously shown above with full length Gal4AD-Pol3 (see section 3.2.1). A "three hybrid" assay was done as described in section 3.2.1 above, using Gal4AD-Pol3CT, LexABD-Cdc1 and myc-tagged Cdc27. The results of this "three hybrid" are shown in Figure 3.4, columns 2-5. The results show that myc-tagged Cdc27 is needed for binding of Gal4AD-Pol3CT to LexABD-Cdc1 and that myc-tagged Cdc27 is not able to cause activation of the reporter gene with either Gal4AD-Pol3CT or LexABD-Cdc1 on their own. Furthermore, the level of β- Gal activity is >100 units, which means that the binding is strong compared to the binding of Gal4AD-Pol3. Therefore, it appears that Gal4AD-Pol3CT is able to bind strongly to LexABD-Cdc1 but only when myc-tagged Cdc27 is present. Myc-tagged Cdc27 appears to have a stabilising effect on the Gal4AD-Pol3CT and LexABD-Cdc1 complex.

A construct of Cdc1 that has the C- terminal 10 amino acids deleted (Cdc1Δ453) was also tested in this "three hybrid" system and the results are shown in Figure 3.4 above, column 6. It appears that whereas full length LexABD-Cdc1 is able to bind to Gal4AD-Pol3CT, LexABD-Cdc1Δ453 is not able to bind, suggesting that the last ten amino acids of Cdc1 are important either for the stability of Cdc1, for binding to Gal4AD-Pol3CT or for the stabilising effect of myc-tagged Cdc27.

3.2.3 ZnF2 alone cannot bind to Cdc1

As shown in section 3.2.2 the C- terminus of Gal4AD-Pol3 (a clone that contained both putative zinc fingers) can bind in the two-hybrid system to LexABD-Cdc1 when myc-tagged Cdc27 is present. In the previous chapter (see section 2.2.6) the second zinc finger was shown to be necessary and sufficient for the binding to the B subunit in *S. cerevisiae*. Therefore, it was decided to investigate whether the second zinc finger on its own is sufficient to bind Cdc1 in *S. pombe*. For this, sequences encoding the second zinc finger of polδ Pol3 (hereafter named ZnF2) were subcloned into

pACT2 (see Figure 3.3 for the sequence of ZnF2; see section 6.2.2.8.4 for subcloning details). pACT2-ZnF2 was co-transformed along with pBTM116-Cdc1 into CTY10-5d, and used in a β- Gal assay as described in section 6.2.2.5. The results of the binding assay are shown in Figure 3.5 below; for raw data see Table 8.6, appendix A.

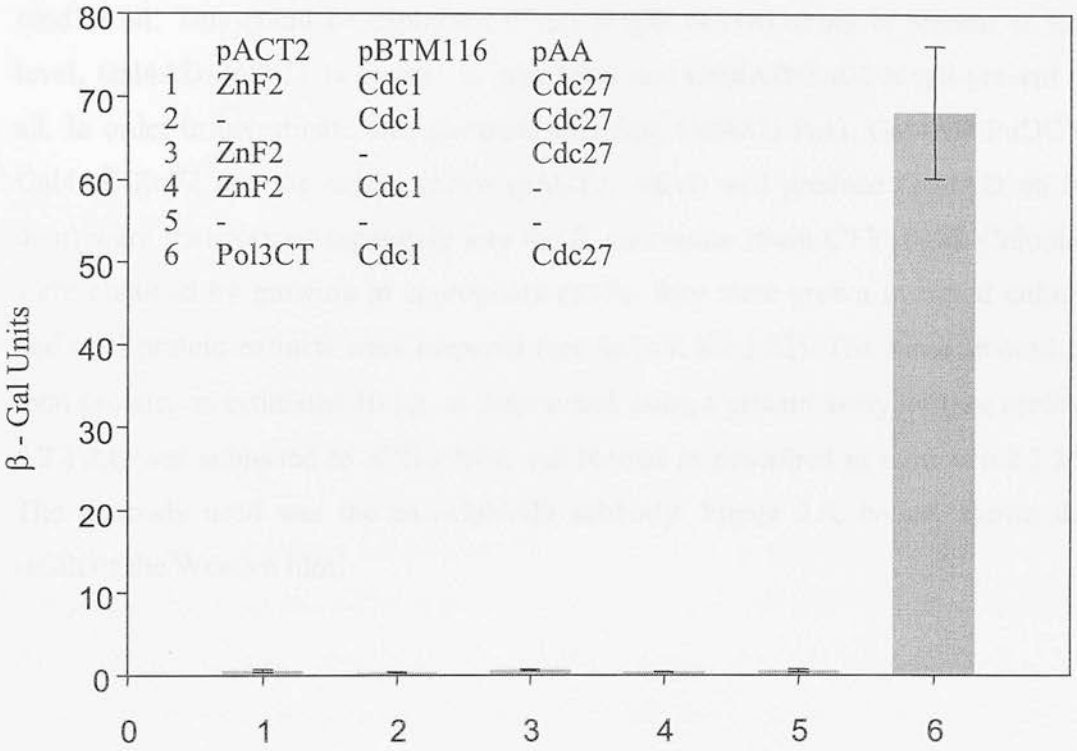

Figure 3.5. β-Gal assay to investigate binding of Gal4AD-ZnF2 to LexABD-Cdc1. "–" indicates that the empty vector was used. Experiments were done in triplicate, column height is the mean of the values and error bars are the standard deviation from the mean.

As can be seen from column 1, it appears that Gal4AD-ZnF2 on its own cannot bind to LexABD-Cdc1, even in the presence of myc-tagged Cdc27. The negative controls (columns 2-5) were as expected, all giving less than one unit of binding and the positive control (Gal4AD-Pol3CT, LexABD-Cdc1 and myc-tagged Cdc27, column 6) again gave strong binding.

3.2.4 Protein expression levels

3.2.4.1 Expression levels of Pol3 proteins

From the work above, it appears that in the two-hybrid system, in the presence of myc-tagged Cdc27, full length Gal4AD-Pol3 is able to bind weakly to LexABD-Cdc1, Gal4AD-Pol3CT is able to bind strongly, whereas Gal4AD-ZnF2 is not able to bind at all. This could be explained if full length Gal4AD-Pol3 is present at low level, Gal4AD-Pol3CT is present at high level and Gal4AD-ZnF2 is not present at all. In order to investigate this, plasmids encoding Gal4AD-Pol3, Gal4AD-Pol3CT, Gal4AD-ZnF2 and the empty vector (pACT2, which will produce Gal4AD on its own) were transformed separately into the *S. cerevisiae* strain CTY10-5d. Colonies were obtained by growing in appropriate media, they were grown in liquid culture and total protein extracts were prepared (see section 6.2.1.22). The same amount of total protein, an estimated 10 μg, as determined using a protein assay kit (see section 6.2.1.23) was subjected to SDS-PAGE and blotted as described in section 6.2.1.25. The antibody used was the anti-Gal4AD antibody. Figure 3.6, below, shows the result of the Western blot.

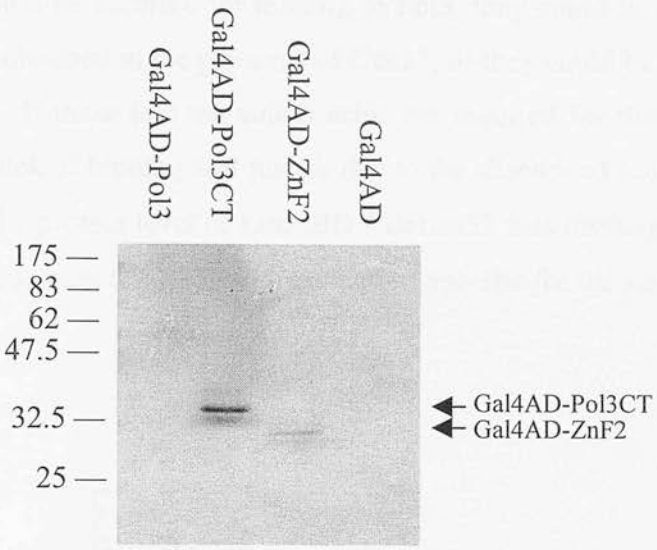

Figure 3.6. Western blot of different Gal4AD-Pol3 fusion proteins. Gal4AD-Pol3, full length Gal4AD-Pol3; Gal4AD-Pol3CT, both Gal4AD-tagged C- terminal Zinc Fingers; Gal4AD-ZnF2, Gal4AD-tagged second C- terminal zinc finger only and Gal4AD, Gal4AD only as produced from the empty vector, pACT. An estimated 10 μg of total protein was loaded in each lane.

As can be seen in Figure 3.6, above, Gal4AD-Pol3CT is produced to a high level, Gal4AD-ZnF2 is produced to a much lower level and Gal4AD-Pol3 and Gal4AD were not produced at a level detectable by the Western blot. The markers used in the Western blot were pre-stained markers, all of which (even the biggest 175kDa in size) were transferred efficiently to the membrane. Hence, it appears that, if present, the Gal4AD-Pol3 would have been transferred efficiently. Longer exposure detected a low level of an approximately 25 kDA protein in the pACT lane but no protein was detected in the Pol3 lane (data not shown). Thus, the low level of β- Gal units obtained with Pol3 could be due to the low level of Pol3 protein. Further, ZnF2 protein has a detectable level of protein.

3.2.4.2 Bait protein levels

In section 3.2.2, above, it was shown that whereas Gal4AD-Pol3CT can bind to LexABD-Cdc1 in the presence of myc-tagged Cdc27 it cannot bind to LexABD-Cdc1Δ453. The following explanations could account for this. The last ten amino

acids of Cdc1 could be required for binding to Pol3, they could be required for the stabilising effect obtained in the presence of Cdc27, or they could be required for the stability of Cdc1. If these last ten amino acids are required for the stability of the protein then the lack of binding will just be due to the absence of protein. In order to investigate this, the protein level of LexABD-Cdc1Δ453 was investigated by doing a Western blot (see section 6.2.1.25) with antibodies specific for the LexABD.

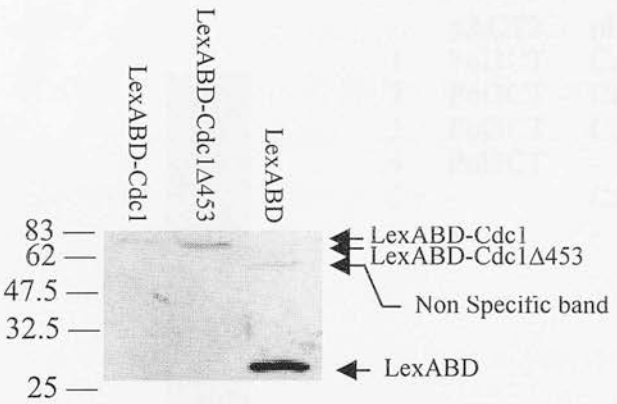

Figure 3.7. Western blot of the bait proteins LexABD-Cdc1 and LexABD-Cdc1Δ453. LexABD is produced from the empty vector pBTM116. An estimated 10 μg of total protein was loaded in each lane.

As can be seen from Figure 3.7, LexABD-Cdc1 is the bait protein with the lowest protein level, LexABD-Cdc1Δ453 has a slightly higher protein level and LexABD (produced from pBTM116) has the highest level of protein. Therefore, it is probable that the lack of binding of LexABD-Cdc1Δ453 to Gal4AD-Pol3, when compared to full length LexABD-Cdc1 is not due to the absence of the LexABD-Cdc1Δ453 protein.

3.2.5 Cdm1 cannot replace Cdc27

As shown above, Gal4AD-Pol3CT can bind to LexABD-Cdc1 but only when myc-tagged Cdc27 is present. It was decided to investigate if Cdm1, the D subunit of polδ, has a similar stabilising effect on the binding of Gal4AD-Pol3CT and

LexABD-Cdc1. To address this, sequences encoding Cdm1 were amplified by PCR and cloned into pAA (see section 6.2.2.8.8 for a more detailed account of the cloning), so the Cdm1 will be produced from the pAA plasmid as a myc-tagged protein. The pAA-Cdm1 plasmid was used in place of pAA-Cdc27 in a similar "three hybrid" assay to those described previously. Results are shown in Figure 3.8 below, see Table 8.6, appendix A for raw data.

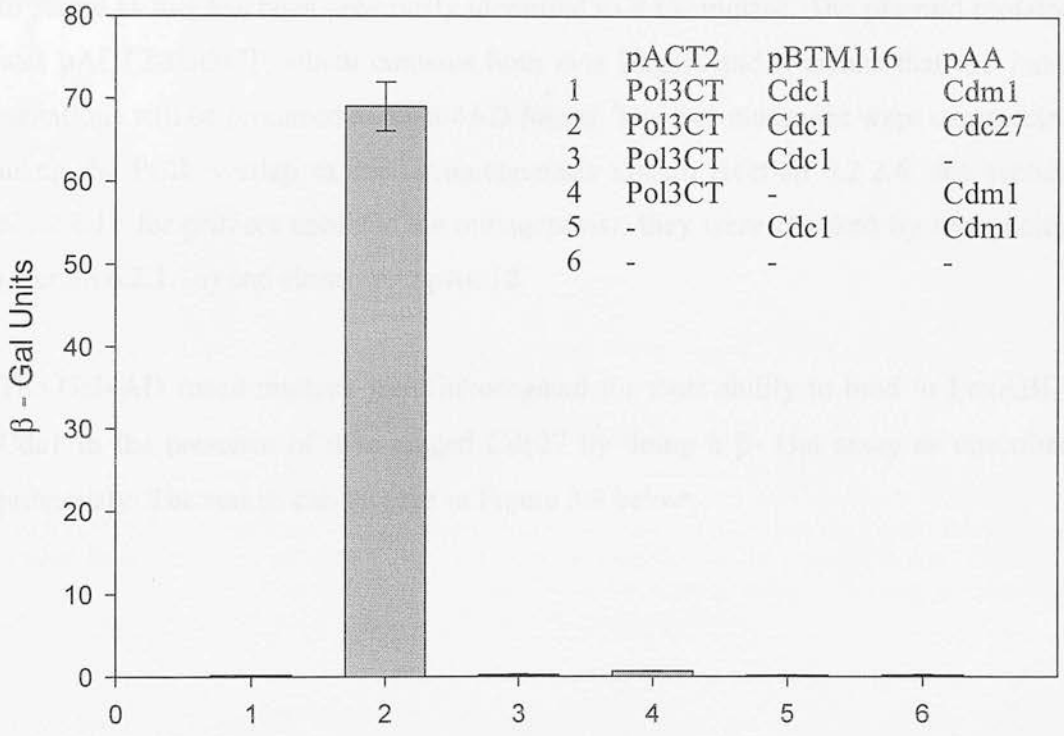

Figure 3.8. Liquid culture β- Gal assay to investigate possible stabilising effect of myc-tagged Cdm1. "–" indicates that the empty vector was used. Experiments were done in triplicate, column height is the mean of the values and error bars are the standard deviation from the mean.

The results show that myc-tagged Cdm1 appears not to be able to stabilise the binding of Gal4AD-Pol3CT and LexABD-Cdc1. Hence, it appears that the stabilising effect is specific to myc-tagged Cdc27.

3.2.6 Mutational Analysis of SpZnF2

In the previous chapter, it was shown by mutational analysis that the co-ordination of the zinc is probably carried out by all four cysteines present in the ZnF2 of *S. cerevisiae* Pol3. It was decided to investigate the four conserved cysteines present in the *S. pombe* Pol3 second zinc finger. For this analysis, five individual mutations were introduced in the second zinc finger of *S. pombe* Pol3. The four cysteines in ZnF2 were all individually mutated to alanine. The fourth cysteine was also mutated to serine as this has been previously identified as a t.s. mutant. The plasmid mutated was pACT2-Pol3CT, which contains both zinc fingers and it means that the ZnF2 mutations will be produced as a Gal4AD fusion. The five mutations were constructed using the PCR overlap extension mutagenesis system (section 6.2.2.6; see section 6.2.2.8.11 for primers used for the mutagenesis), they were checked by sequencing (section 6.2.1.16) and cloned into pACT2.

The Gal4AD fused mutants were investigated for their ability to bind to LexABD-Cdc1 in the presence of myc-tagged Cdc27 by doing a β- Gal assay as described previously. The results can be seen in Figure 3.9 below.

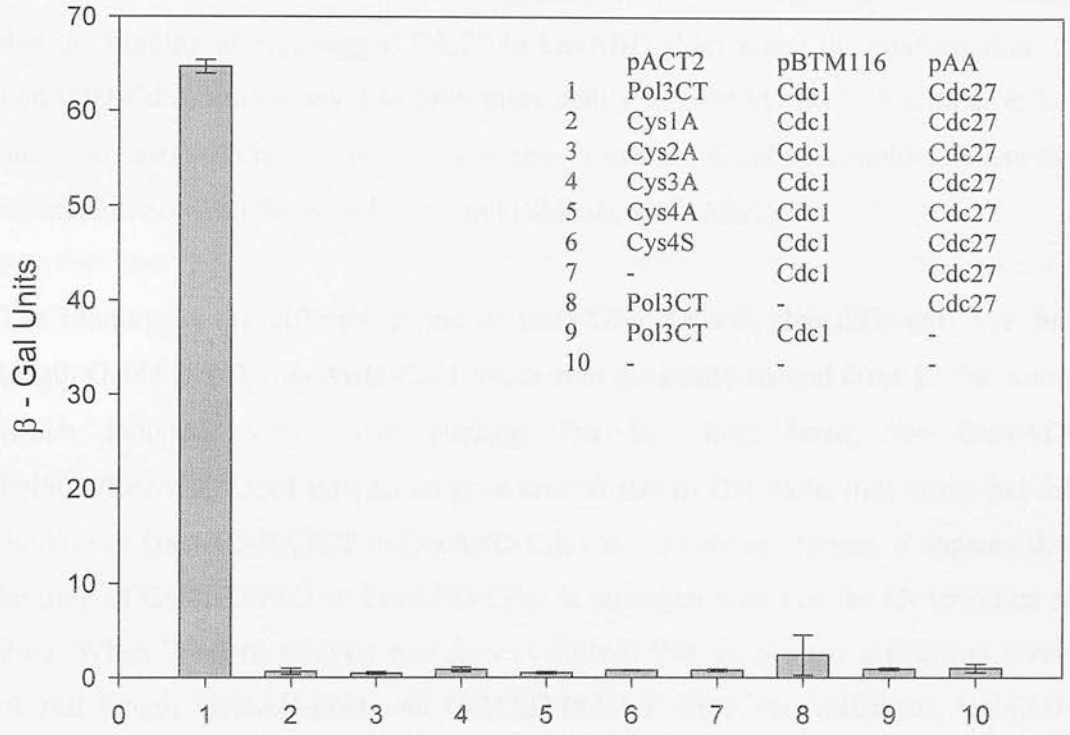

Figure 3.9. Liquid culture β- Gal assay to investigate the binding of Gal4AD-ZnF2 mutants to LexABD-Cdc1. "–" indicates that the empty vector was used. Experiments were done in duplicate, column height is the mean of the values and error bars are the standard deviation from the mean. Cys1A is the mutation of the first cysteine to alanine; Cys2A is the mutation of the second cysteine to alanine and so on. Cys4S is the mutation of the fourth cysteine to serine.

It appears that none of the Gal4AD-mutants are able to bind to LexABD-Cdc1, whereas the wild type is able to, as shown previously. Further, all the negative controls produce less than one unit of β- Gal activity as expected.

3.3 Discussion

In this chapter it was demonstrated that, in the two-hybrid system, Gal4AD-Pol3 (both full length and Pol3CT) is not able to bind independently to LexABD-Cdc1. This was surprising as it was previously shown that Pol3 is able to bind to Cdc1 *in vitro* and that in *S. cerevisiae* the C- terminus of Pol3 can bind to the B subunit (Pol31), although, as discussed previously, the endogenous C subunit (Pol32) may be involved in the binding. Interestingly, in the presence of myc-tagged Cdc27 both full

length Gal4AD-Pol3 and Gal4AD-Pol3CT are able to bind to LexABD-Cdc1. Cdc27 is able to bind to Cdc1 but not to Pol3 (MacNeill *et al.,* 1996), therefore, it is likely that the binding of myc-tagged Cdc27 to LexABD-Cdc1 alters the conformation of LexABD-Cdc1 and causes it to bind more stably to Gal4AD-Pol3. A similar effect has been seen with human polδ. It was shown that the C subunit could stabilise the interaction between the A and B subunit (Shikata *et al.,* 2001).

The binding of the different forms of Gal4AD-Pol3 was also different. The full length Gal4AD-Pol3/LexABD-Cdc1 interaction gave only around three β- Gal units, which indicates very weak binding. On the other hand, the Gal4AD-Pol3CT/LexABD-Cdc1 interaction gave around 100 β- Gal units, indicating that the binding of Gal4AD-Pol3CT to LexABD-Cdc1 is very strong. Hence, it appears that binding of Gal4AD-Pol3 to LexABD-Cdc1 is strongest with just the C- terminus of Pol3. When Western analysis was done it showed that the protein expression levels of full length Gal4AD-Pol3 and Gal4AD-Pol3CT were very different. Gal4AD-Pol3CT was present at a high level, whereas the protein level of full length Gal4AD-Pol3 could not be detected on the Western blot. The significant difference in binding can therefore be explained: full length Gal4AD-Pol3 produces less β- Gal units because there is less of it present, when compared to Gal4AD-Pol3CT.

The investigation was then continued with the aim to define the binding region of Pol3 even further. In the previous chapter, it was shown that in *S. cerevisiae* the second zinc finger of Pol3 is sufficient to bind to the B subunit. Therefore, the binding of ZnF2, the second zinc finger of Pol3 to the *S. pombe* B subunit (Cdc1) was investigated. However, when the experiments were carried out, it was discovered that the Gal4AD-ZnF2 does not bind to LexABD-Cdc1, even in the presence of myc-tagged Cdc27. This was very surprising given that this is the case in *S. cerevisiae*. This unexpected result can mean a number of things. 1) The binding of Gal4AD-Pol3 to LexABD-Cdc1 in *S. pombe*, unlike *S. cerevisiae*, is via the first zinc finger. 2) The binding requires a fragment of Pol3 that is longer than Gal4AD-ZnF2. 3) The binding to Gal4AD-ZnF2 on its own is too weak to be detected by a two-

hybrid assay. 4) The Gal4AD-ZnF2 on its own is unstable and is degraded, and hence, no protein is present in the assay.

Western analysis however, has ruled out one of the four possibilities described above. It was shown by western analysis that even though Gal4AD-Pol3CT was produced to a higher level, Gal4AD-ZnF2 was produced to a detectable level. It is possible that the level of protein produced is insufficient to produce a level of binding detectable in the β- Gal assay. However, Gal4AD-Pol3 did not produce a level of protein that was detectable by Western analysis but did show weak binding in the β- Gal assay. Hence, it appears that Gal4AD-ZnF2 is present in the assay at a level that would allow a detectable interaction and therefore, it appears that possibility "4) The Gal4AD-ZnF2 on its own is unstable and is degraded, and hence no protein is present in the assay" is not correct.

Possibility "1) The binding of Gal4AD-Pol3 to LexABD-Cdc1 in *S. pombe*, unlike *S. cerevisiae*, is via the first zinc finger." is also probably not the case. In the mutational analysis of the ZnF2 it was discovered that mutation of any of the cysteines in the ZnF2 region completely abolished binding of Gal4AD-Pol3CT to LexABD-Cdc1. Further, even if the mutation was to another nucleophilic amino acid, as in the mutation from a cysteine to a serine, it still completely abolished binding. This suggests that either the second zinc finger is indeed important for binding to LexABD-Cdc1 or that the Gal4AD-ZnF2 mutations are affecting the first zinc finger, that is involved in the binding. As zinc fingers are usually independent domains it is likely that a change of the structure of one zinc finger will not affect the other. Hence, it is likely that the second zinc finger is involved in the binding and that the cysteines affect binding, probably by maintaining the structure of the ZnF2 region.

Therefore, suggestions "2) The binding requires a fragment of Pol3 that is slightly longer than Gal4AD-ZnF2" and "3) The binding to Gal4AD-ZnF2 on its own is too weak to be detected by a two-hybrid assay" remain. *In vitro* experiments performed by Dr. L. Ciufo, a postdoctoral fellow in the lab, have helped to elucidate this. The *in vitro* assay investigated the binding of Pol3CT and ZnF2 to Cdc1. Briefly, the

method involved the production in *E. coli* of recombinant His6 tagged Pol3CT or ZnF2 and the mixing with ^{35}S radioactively labelled *in vitro* produced Cdc1. After mixing, Nickel agarose slurry was added which will bind to the His6 tag. If His6-Pol3CT or His6-ZnF2 is able to bind to ^{35}S-Cdc1 the supernatant will be depleted of radioactivity and it will be present in the pellet. See caption of Figure 3.10 for more details. The results are shown in Figure 3.10.

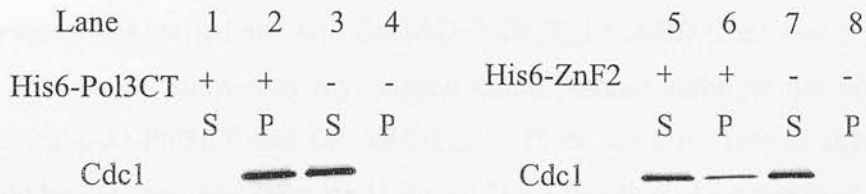

Figure 3.10. *In vitro* binding of His6-Pol3CT and His6-ZnF2 to ^{35}S-Cdc1. Both Pol3CT and ZnF2 were expressed as recombinant His6 tagged proteins from the pQE vector in M13 (pREP4), an *E. coli* strain. For this, liquid cultures were grown and expression was induced at 37°C overnight by the addition of IPTG to 1 mM once the OD_{600} of the culture reached 0.7. Purification was achieved by incubating with Ni agarose and washing twice in buffer containing 8 M urea pH 6.3, before eluting with buffer containing 8 M urea pH 4.5. The proteins were refolded by dialysing overnight into 50 mM borate buffer pH 8.0. A sample of the refolded protein was mixed with radioactive ^{35}S-Cdc1 produced using the *in vitro* coupled translation and transcription reticulocyte reaction (Promega). This was mixed on a wheel and after 30 min incubation 80 µl of 50% Ni agarose slurry was added and incubated for an extra 90 min. A control in which there is no His-Pol3CT or no His6-ZnF2 was also done and was treated as the other samples (lanes 3, 4, 7 and 8). Samples were then spun down, the supernatant was TCA precipitated and run on a gel, labelled "S" above. The pellet was washed and heated at 65°C before being run on a gel, labelled "P" above. The gel was run, fixed, stained and de-stained and dried down on blotting paper. The dried gel was exposed overnight on MR film. Film is shown above. Figure courtesy of Dr. Leo Ciufo.

The controls (lanes 3, 4, 7, and 8) show that when there is no His tagged protein present the Cdc1 is only present in the supernatant, indicating that the Ni agarose slurry does not cause Cdc1 to be pulled down. The *in vitro* results confirm that Pol3CT is able to bind to Cdc1 (lane 2) and in the *in vitro* studies this binding was shown not to need Cdc27. The *in vitro* results also show that ZnF2 on its own can bind to Cdc1 (lane 6) and they also show that this binding is weaker than that of Pol3CT, as some Cdc1 is detectable in the supernatant (lane 5).

This indicates that of the possibilities suggested above to explain the two-hybrid result, the one that is most likely is "3) The binding to ZnF2 on its own is too weak to be detected by a two-hybrid assay". However, it is possible that the two-hybrid interaction would be stronger with a ZnF2 fragment that is longer than that used in the above studies. Nonetheless, ZnF2 is, *in vitro*, sufficient to bind to Cdc1.

The stabilising effect of myc-tagged Cdc27 was intriguing and led to an investigation into whether the D subunit of the complex, Cdm1, has a similar stabilising effect. A binding assay was carried out with Gal4AD-Pol3CT, LexABD-Cdc1 and myc-tagged Cdm1. The results show that myc-tagged Cdm1 cannot stabilise the interactions between Gal4AD-Pol3CT and LexABD-Cdc1. There are a number of reasons why this could be the case. For example 1) It could be a function of myc-tagged Cdc27 to stabilise the Gal4AD-Pol3/LexABD-Cdc1 interaction, and this cannot be achieved by any other protein. 2) Any protein that binds to Pol3CT or Cdc1 can stabilise the interaction but Cdm1 does not bind to Pol3CT or Cdc1. 3) Cdm1 might bind very weakly and therefore cannot stabilise the complex. 4) Cdm1 is quickly degraded so it cannot stabilise the interaction. This last possibility could be investigated by Western analysis. Identifying the Cdm1 binding partner/partners within polδ may help to speculate with a greater degree of certainty, at present any number of reasons could explain the situation.

The difference between the strength of binding of Gal4AD-Pol3CT to LexABD-Cdc1 and LexABD-Cdc1Δ453 was striking. Western analysis has shown that LexABD-Cdc1Δ453 is expressed at a higher level than LexABD-Cdc1. Therefore, it is very likely that the difference in binding is not due to the difference in protein expression levels. Two reasons could account for this difference in binding. One is that the last ten amino acids are directly involved in the binding to Gal4AD-Pol3CT. The other is that these amino acids could be involved in the stabilising effect observed with myc-tagged Cdc27. Work by MacNeill *et al.*, (1996) in which a two-hybrid analysis was done with Gal4AD-Cdc27 and LexABD-Cdc1Δ453 it was shown that Gal4AD-Cdc27 was able to bind to LexABD-Cdc1Δ453 approximately

three times better than it was able to bind to LexABD-Cdc1. The Western analysis performed in this chapter has shown that the protein level of LexABD-Cdc1Δ453 is approximately three times higher that that of LexABD-Cdc1 thereby explaining the increased binding to Gal4AD-Cdc27. Hence, it appears that LexABD-Cdc1Δ453 is able to bind to Cdc27 so its inability to bind to Gal4AD-Pol3CT probably indicates that the last ten amino acids of Cdc1 are involved in the binding to Pol3.

In summary, the results presented in this chapter show that Gal4AD-Pol3 and LexABD-Cdc1 are able to interact, but only in the presence of myc-tagged Cdc27, and that myc-tagged Cdc27 cannot be replaced by myc-tagged Cdm1. Also, the region of Pol3 needed to interact with Cdc1 was narrowed down, using a two-hybrid approach, to the C- terminal two zinc fingers. Mutations within the second zinc finger abolish the Gal4AD-Pol3/LexABD-Cdc1 interaction indicating that this zinc finger is needed for binding. Furthermore, Dr. Leo Ciufo has performed *in vitro* analysis that suggests that the second zinc finger on its own is sufficient to bind to Cdc1. Hence, the interaction of Pol3 with Cdc1 has been narrowed down to the C-terminus of Pol3, most likely to the second zinc finger. Further, the last ten amino acids of LexABD-Cdc1 have been found to be important for binding to Gal4AD-Pol3CT.

4 Mutational Analysis of Cdc1

4.1 Introduction

Little is understood about the precise protein-protein interactions that take place within pol δ. Although it is clear that Pol3 interacts with Cdc1, and Cdc1 interacts with Cdc27 (MacNeill *et al.,* 1996), the precise regions involved in these interactions remain undefined. The subunit which has been most extensively studied is Cdc27 which is known to interact with Cdc1 via its N- terminal 160 amino acids. It is also known to interact with PCNA, outwith the complex, via the PCNA binding motif located in its C- terminus (Reynolds *et al.,* 2000). In the previous chapter, using a combination of two-hybrid and mutational analysis the interactions of Pol3 with Cdc1 were narrowed down to the C-terminus of Pol3, to the ZnF2 region.

This chapter describes how extensive mutational analysis of the *cdc1*$^+$ gene was performed using two methods: one to generate random mutants and the other to generate site directed mutants. It was hoped that this would yield further information on the binding of Cdc1 to the other members of the complex and on the possible function of Cdc1.

4.2 Results

4.2.1 Mutagenesis of Cdc1

Two methods were used for the mutagenesis of *cdc1*$^+$, a random insertion mutagenesis method and a site directed method. To generate random mutants the pentapeptide insertion mutagenesis method was utilised and is described by Hallet *et al.*, (1997a), Cao *et al.*, (1997b) and Hayes *et al.*, (1997c) and in section 6.2.2.1. In brief, it is a transposon-based insertion mutagenesis system. One *E. coli* strain (FH1046) containing the transposon Tn*4430*Ω5 (see Mahillon and Lereclus, (1988) for details of Tn*4430*Ω5) was transformed with a plasmid (pBR322) containing the gene of interest (in this case, *cdc1*$^+$ cDNA). In order to ensure that the mutations

were independent, several independent transformants were used and they were mated to another *E. coli* strain (DS941). After mating, cells were grown in media that selects for the DS941 strain, the transposon and pBR322-Cdc1 (see section 6.2.2.1). This mating and selection allows for selecting for transposons that have inserted into the plasmid of interest (pBR322-Cdc1) and not anywhere else, for example in the DNA of the FH1046 strain. Also, the DS941 strain allows for the resolving of the cointegrate formed when the transposon inserts (Hallet *et al.,* 1997a).

As the insertion of the transposon in the plasmid is random, there is the need to screen for plasmids in which the insertion occurred in the gene of interest and not anywhere else in the plasmid. This screening was performed as follows. The cells from the mating were grown in liquid culture and the plasmid was extracted (see section 6.2.1.5). Then restriction digest with enzymes flanking $cdc1^{+}$ was performed. The occasions in which the transposon has inserted into *cdc1* will show a marked size increase, compared to the wild type gene, as the transposon is approximately 4kb in size. After screening, the bulk of the transposon was deleted utilising two unique restriction enzyme sites (*Kpn*I sites) located near the ends of the transposon and self-ligating the resulting plasmid. The end result is the in-frame insertion of 15bp into *cdc1*. Five of these bases are due to target site duplication and 10 are due to the sequence of the transposon. Once expressed the Cdc1 protein will have a five amino acid insertion and the identity of these amino acids will depend partly on the location of the transposon insertion site and on the sequence of the Tn44330 transposon itself. See Figure 4.1 and Figure 4.2 for a diagram of the pentapeptide insertion mutagenesis system and the end result options.

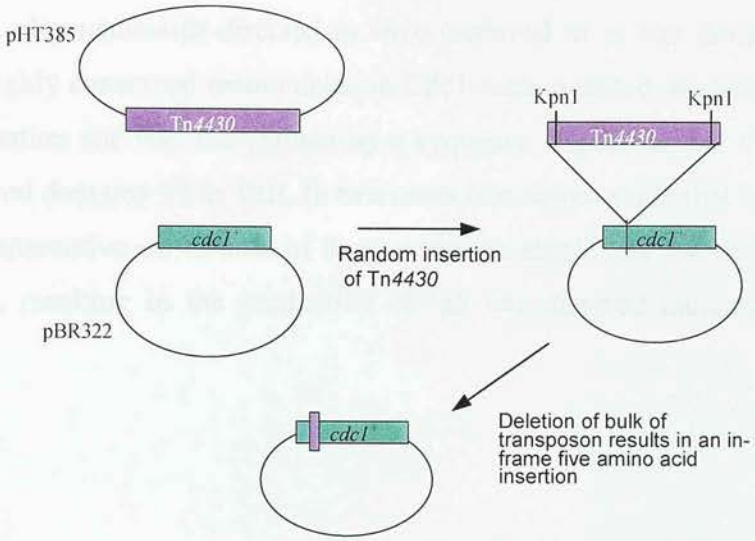

Figure 4.1. Diagram of the pentapeptide insertion mutagenesis system. After insertion of the transposon, restriction digest with KpnI results in the in frame insertion of five amino acids. See text for further details.

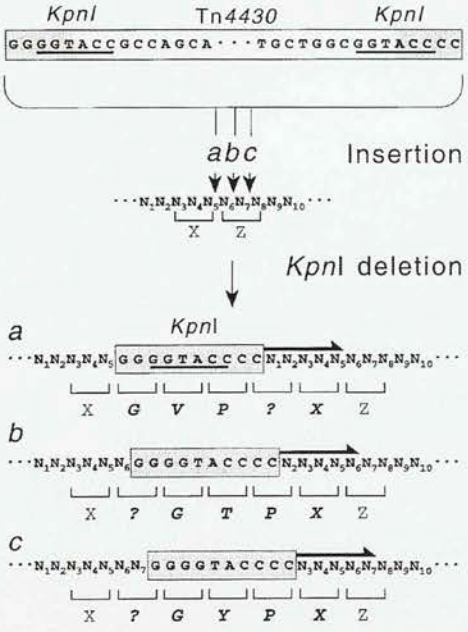

Figure 4.2. The different possibilities of the pentapeptide insertion mutagenesis system. Rectangle is sequence from Tn*44330*, bases underlined are the *Kpn*I site. Arrow indicates the five base pair target site duplication. Letter under the sequences indicate amino acids in single letter code. "X" and "Z" are the target site amino acids. The identity of the amino acid "?" will depend on the identity of the bases in which it inserts. If the insertion is at position c of a tyrosine a stop codon will be generated. From (Hallet *et al.*, 1997a)

For the oligonucleotide-directed *in vitro* (referred to as site directed) mutagenesis, eight highly conserved amino acids in Cdc1 were mutated to alanine. The choice of the mutation site was determined by a sequence alignment and they all fell within conserved domains VI to VIII. In two cases two amino acids that were to be mutated were consecutive, so in both of these cases the single and the double mutants were created, resulting in the production of ten site directed mutants. See Figure 4.3 below.

Figure 4.3. Location of the site directed (A) mutants. Boxes indicate mutation sites. Boxes with Roman numerals indicate ten conserved boxes identified in a previous study (Reynolds and MacNeill, 1999). Figure adapted from Figure 1.3, chapter 1.

All the mutants, both random and site directed, were subcloned into pREP3xH_6BN so Cdc1 will be expressed as an MRGS(H_6) fusion under the control of the nmt1 promoter. Altogether, the site directed and pentapeptide mutagenesis yielded a total of 41 mutants, ten site directed and 31 pentapeptide mutants (ten of which were generated by E. Knatko, a Summer student in the lab). The mutants were labelled according to their type. Site directed mutants were labelled A mutants, ranging from A1 to A10 (where A9 is the double mutant of A1 and A2; A10 is the double mutant of A5 and A6). The pentapeptide insertion mutants generated in this study were labelled J mutants, and ranged from J1 to J22 (there is no J14). The ten pentapeptide insertion mutants generated by E. Knatko (see above) were labelled E mutants, ranging from E1 to E10. See Figure 4.4 for a diagramatic representation of the location of both sets of mutations (random and site directed). Table 4.1, below shows the amino acid position at which each mutation is located and, in the case of the pentapeptide insertion mutants, the amino acids inserted.

Mutant	Mutation	In sub-domain	Same as	Amino Acids Inserted
A1	Pro292⇨Ala292	VI		
A2	Gly293⇨Ala293	VI		
A3	Asp296⇨Ala296	VI		
A4	Pro303⇨Ala303	VI		
A5	Asn328⇨Ala328	VII		
A6	Pro329⇨Ala329	VII		
A7	Gly344⇨Ala344	VII		
A8	Pro374⇨Ala374	VIII		
A9	A1 and A2	VI		
A10	A5 and A6	VII		
J1	Insertion at 141	III		GlyValProLeuSer
J2	Insertion at 25	I	E10,J18, J20	ArgGlyThrProTyr
J3	Insertion at 62	I/II	E3, E8	GlyValProLeuLeu
J4	Insertion at 127	III	J5, J13	GlyValProArgIle
J5	Insertion at 127	III	J4, J13	GlyValProArgIle
J6	Insertion at 124	III		GlyValProHisTyr
J7	Insertion at 393	VIII		GlyValProLeuMet
J8	Insertion at 83	II	J19	GlyValProLeuLys
J9	Insertion at 174	III/IV		ArgGlyThrProMet
J10	Insertion at 77	II	J11, E6, E7	ArgGlyThrProTyr
J11	Insertion at 77	II	J10, E6, E7	ArgGlyThrProTyr
J12	Insertion at 7	/I		GlyValProHisTyr
J13	Insertion at 127	III	J4, J5	GlyValProArgIle

J15	Insertion at 203	IV/V		GlyValProLeuGln
J16	Insertion at 160	III/IV		GlyGlyThrProVal
J17	Insertion at 28	I		**End**GlyTyrProGln
J18	Insertion at 25	I	E10, J2, J20	ArgGlyThrProTyr
J19	Insertion at 83	II	J8	GlyValProLeuLys
J20	Insertion at 25	I	E10, J2, J18	ArgGlyThrProTyr
J21	Insertion at 275	V/VI		GlyValProGlnLeu
J22	Insertion at 212	IV/V		GlyValProLeuArg
E1	Insertion at 66	I/II		GlyValProGlnSer
E2	Insertion at 25	I		16bp Insertion(=**End**)
E3	Insertion at 62	I/II	E8, J3	GlyValProLeuLeu
E4	Insertion at 10	/I		GlyValProProCys
E5	Insertion at 86	II		MetGlyTyrProVal
E6	Insertion at 77	II	E7, J10, J11	ArgGlyThrProTyr
E7	Insertion at 77	II	E6, J10, J11	ArgGlyThrProTyr
E8	Insertion at 62	I/II	E3, J3	GlyValProLeuLeu
E9	Insertion at 124	III		GlyGlyTyrProTyr
E10	Insertion at 25	I	J2, J18, J20	ArgGlyThrProTyr

Table 4.1 Cdc1 mutation insertion sites. "A" mutants are site-directed and "J" and "E" mutants are pentapeptide insertion mutants. Numbers are amino acid number where the mutation is located. "Same as" indicates if another mutant was found at the same amino acid number as hotspots for mutations were found, see text for more details. None of the site directed mutants are repeated. "Amino acids inserted" indicate the amino acids inserted for the pentapetide insertion mutants, "**End**" indicates a stop codon. In the case of E2 sixteen base pair were inserted that introduced a frameshift and a stop codon was hence in frame. "I" indicates that the mutation is in conserved region I; "I/II" indicates that the mutation lies in between conserved regions I and II; "/I" indicates that the mutation lies before conserved region I.

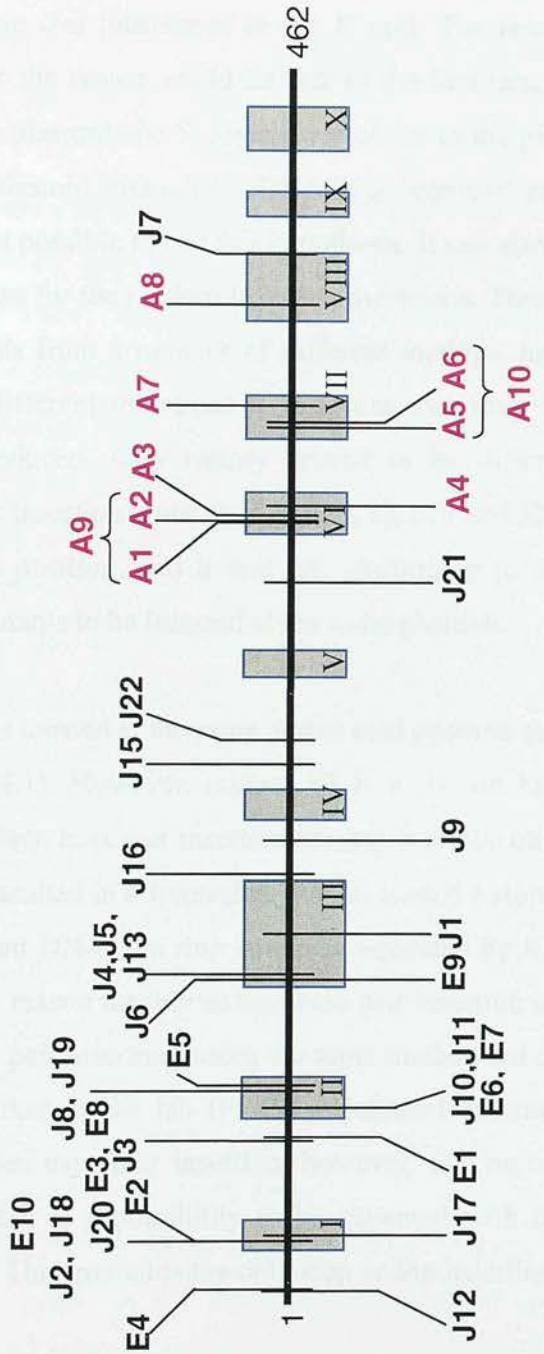

Figure 4.4. Diagrammatic representation of Cdc1 with the location of the mutations. Lines indicate mutation sites. Boxes with Roman numerals indicate ten conserved boxes identified in a previous study (Reynolds and MacNeill, 1999). Numbers indicate amino acid numbers. "A" mutants are site directed whereas "J" and "E" mutants are pentapeptide insertion mutants. A9 is the double mutant of A1 and A2. A10 is the double mutant of A5 and A6. Site directed mutants are in pink and pentapeptide mutants are in black.

As can be seen from Table 4.1 and Figure 4.4 the pentapeptide insertion method is not entirely random. Most of the mutations were introduced in the 5' end of *cdc1*$^+$ and only one was introduced in the 3' end. The reason for this is unclear. It was thought that the reason could be due to the fact that, due to the orientation of the insert in the plasmid, the 5' region was closer to the pBR322 promoter (p4). Cloning to create a plasmid with *cdc1*$^+$ cDNA in the opposite orientation proved unsuccessful so it was not possible to test this hypothesis. It can also be observed that there appear to be hotspots for the random insertion mutations. These mutations were independent transformants from a number of different matings, hence, it was thought that they would be different mutations if insertion was truly random. However, of the 31 mutants produced, only twenty proved to be different. In some instances four independent insertion mutants (e.g. E10, J2, J18 and J20) were found to be insertions at the same position, and it was not uncommon to find two or three independent insertion mutants to be inserted at the same position.

Mutant E2 is located at the same amino acid position as mutants E10, J2, J18 and J20 (see Table 4.1). However, mutant E2 is a sixteen base pair insertion and not the expected fifteen base pair insertion present in all the other pentapeptide mutants. This extra base resulted in a frameshift, which caused a stop codon (UAG) to be in frame. In this mutant DNA, the stop codon is separated by 87 bp from another stop codon (UAA). The reason for this sixteen base pair insertion is not clear, however, a similar sixteen base pair insertion, using the same method but on a different gene, was found by a co-worker in the lab (F. Gray, personal communication). Mutant J17 has a normal fifteen base pair insertion, however, this mutant has a stop codon inserted (UAG), which is a possibility to be expected with this mutagenesis method (see Figure 4.2). This mutant is the only stop codon insertion in all the mutants isolated in this study.

In total, this study has generated 20 different pentapeptide insertion mutants and 10 different site directed mutants. When taken together these mutations provide a good coverage of the length of the Cdc1 gene.

4.2.2 Can the mutants rescue cdc1Δ?

An assay was set up in which the ability of the Cdc1 mutants to rescue an *S. pombe* strain, deleted for *cdc1*$^+$ was investigated in order to be able to determine whether the mutants had a detrimental effect.

The assay was carried out as follows. Since Cdc1 is essential, a diploid *S. pombe* strain in which one copy of *cdc1*$^+$ is replaced with *ura4*$^+$ marker was used (*cdc1::ura4*$^+$/*cdc1*$^+$ *leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-M210/M216 h*$^-$/*h*$^+$, which will be referred to as *cdc1Δ*). All the mutant pREP3xH_6BN-Cdc1 plasmids were transformed into this strain by electroporation (see section 6.2.1.6) and presence of the plasmid was selected for, by growth in the absence of leucine. The transformants were then allowed to sporulate by growing on malt extract (see section 6.2.1.8). Each transformant will yield four haploid spores: two with a wild type copy of *cdc1*$^+$ and two with the *ura4*$^+$ marker replacing *cdc1*$^+$. By allowing the cells to germinate in media lacking uracil (EMM+A) only cells that have the *cdc1*$^+$ gene deleted and replaced with *ura4*$^+$ will be able to grow. If however, the cells are grown in medium containing uracil and adenine (EMM+U+A) then all spores will be able to grow. In order to decide if any given *cdc1* mutant was able to support growth the following was carried out. The spores were allowed to germinate in media both with uracil (EMM+U+A) and without added uracil (EMM+A). If the *cdc1* mutant contained in the plasmid is able to rescue, i.e. is able to support growth of the *cdc1Δ* strain, a high number of colonies will be expected on both the EMM+U+A plate (where all spores are able to grow) and on the EMM+A plate. If however, the *cdc1* mutant contained in the plasmid is not able to rescue, i.e. is not able to support growth of the *cdc1Δ*, then a high number of colonies will be expected on the EMM+U+A plate (as the non-disrupted copy of *cdc1*$^+$ will be able to support growth) but no colonies will be expected on the EMM+A plate.

After sporulation of the diploids, but before germination, the spores were treated with helicase (see section 6.2.1.8) which results in the killing of vegetative cells. In theory this results in the destruction of all remaining diploids, but in practice it does not. Also, it is possible that diploid spores might be present which will survive the

helicase treatment and will germinate to produce diploid cells. This raises the problem of the presence of diploid cells which contain mutant pREP3xH_6BN-Cdc1 but which will also contain a wild type copy of *cdc1*$^+$, are ura$^+$ and will, therefore, grow on medium lacking uracil (EMM+A) regardless of whether the Cdc1 mutant is able to rescue the *cdc1Δ* strain. To overcome this problem the fact that the diploids would be ade$^+$ (and hence, able to grow in medium lacking adenine) whereas the haploid would not, was used. Also, diploids would be able to sporulate (so they will produce spores on ME media, see section 6.1.6.2) whereas haploids would not. Hence, by checking that the cells that are able to grow in EMM were haploid it could be confidently stated whether the mutation was able to rescue or not. When this protocol was followed it was typical to find more than 1000 colonies on the EMM+U+A plates and the EMM+A plates of the mutants that were able to rescue. On the EMM+A plates containing those mutants that were not able to rescue it was typical to find less than 100 colonies. A sample of these colonies were checked and were found to be diploid. In this screen all of the cells were grown at 32°C.

The plasmid used in this screen was pREP3xH_6BN, where the production of wild type Cdc1 and the mutants are under the control of the nmt1 (nmt = *n*o *m*essage *t*hiamine) promoter which enables control of the level of protein expression by the presence or absence of thiamine. In the absence of thiamine the promoter is derepressed which results in the protein being overexpressed. In the presence of thiamine, the promoter is repressed, which results in the protein being expressed to a lower level. (It should be noted that the expression levels of the mutants in comparison with wild type was not established.) Each mutant was investigated for its ability to rescue, as described above, both with the promoter derepressed and with the promoter repressed. All the mutants were tested at least twice to be sure of the results.

This ability to alter the levels of protein expression, gave rise to a way of classifying the rescue by mutants into three categories: a) Mutants that do not rescue at any expression level; b) Mutants that rescue when overexpressed (promoter derepressed) but do not rescue when expressed to a lower level (promoter repressed); and c)

Mutants that rescue at any expression level. Figure 4.5 shows the mutants and their ability to rescue. White boxes indicate rescue at all protein levels, black boxes indicate no rescue at any level and half filled boxes indicate rescue only when the mutant is overexpressed but not when expressed to levels closer to wild type (see Table 8.9 in appendix A for a more detailed account of the ability to rescue).

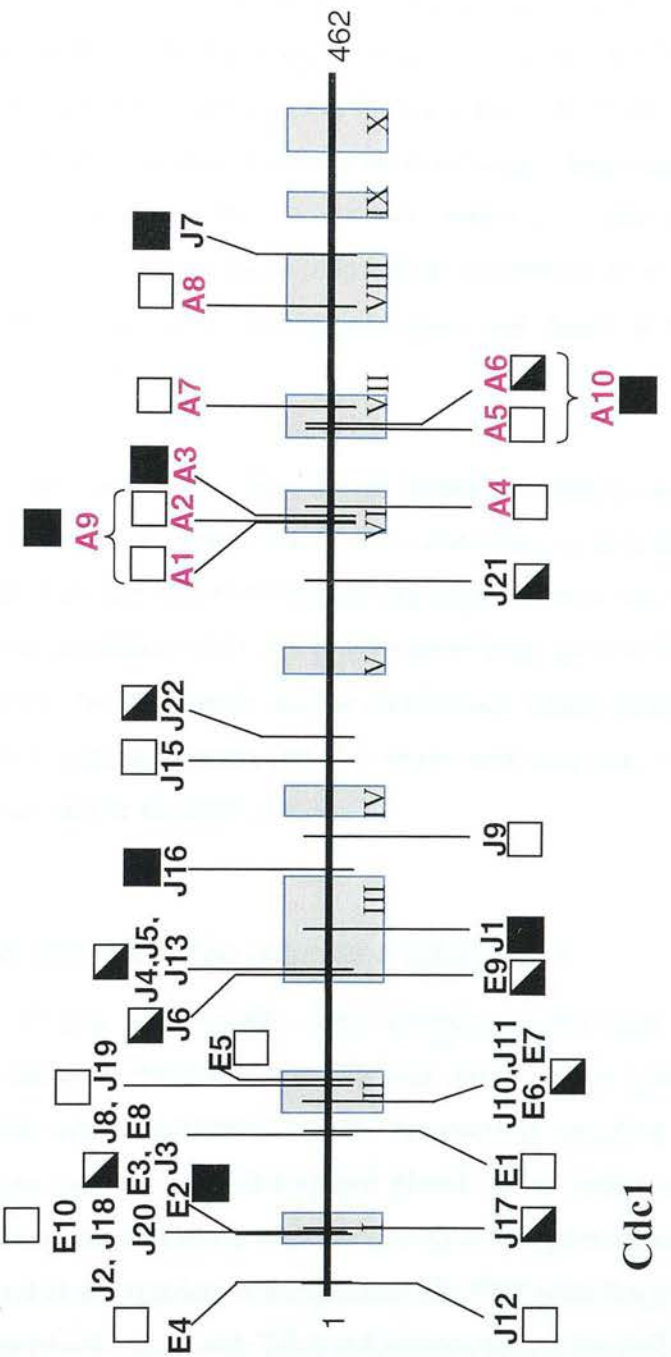

Figure 4.5. Ability of the Cdc1 mutants to rescue *cdc1Δ*. Lines indicate mutation sites. Boxes with Roman numerals indicate ten conserved boxes identified in a previous study (Reynolds and MacNeill, 1999). Numbers indicate amino acid numbers. "A" mutants are site directed whereas "J" and "E" mutants are pentapeptide insertion mutants. A9 is double mutant of A1 and A2. A10 is double mutant of A5 and A6. Site directed mutants are in pink and pentapeptide mutants are in black. White boxes indicate rescue at all protein levels. Filled boxes indicate no rescue at any level and half filled boxes indicate rescue only when mutant is overexpressed but not when expressed to levels closer to wild type. See text for further details.

Figure 4.5 shows that a high number of pentapeptide insertion mutants (eight out of the twenty) are able to rescue at all protein levels. This is very surprising as it suggests that an insertion of five amino acids in Cdc1 does not have any effect on the protein, as it can rescue a deletion. On the other hand, there are four mutants that are not able to rescue under any protein level expressed. These mutants are E2, J1, J16 and J7. It was very surprising to see that mutant J17 was able to rescue when overexpressed. This mutant has a stop codon introduced so it is expected that the protein produced only be 28 amino acids long and hence, it was expected that it would be unable to rescue.

The site directed mutants also show a high number of mutants (six out of the ten) that are able to rescue at all protein levels. It is interesting to note that in both cases the double mutants are not able to rescue but the single mutants are, although in one case only when overexpressed (A6). A3 is very interesting, as it is the only single amino acid substitution (of an aspartic acid to an alanine), which results in the protein not being able to rescue, suggesting that this amino acid may play a very important role in the structure and/or function of Cdc1.

4.2.3 Can the mutants rescue a cdc1 *t.s.?*

The Cdc1 "J" and "A" mutants were investigated for their ability to rescue a temperature sensitive strain of *cdc1*. This was carried out as follows. The mutants in pREP3xH_6BN were transformed into the temperature sensitive strain *cdc1-P13* by electroporation (see section 6.2.1.6) and plated out in different conditions: at the permissive temperature (28°C) with the promoter derepressed or with the promoter repressed; and at the restrictive temperature (36.5°C) with the promoter derepressed or with the promoter repressed. Table 4.2, below, shows the ability of the mutants to rescue at the restrictive temperature (36.5°C) with the promoter derepressed or repressed (tables 8.10 and 8.11 in appendix A, give further details of their ability to rescue).

Ability of the Cdc1 "J" and "A" mutants to rescue a *cdc1* t.s. strain at the restrictive temperature (36.5 °C)					
Mutant	*cdc1-P13* rescue?		Mutant	*cdc1-P13* rescue?	
	Promoter derepressed	Promoter repressed		Promoter derepressed	Promoter repressed
3x	-	-	J1	-	-
Cdc1	+	+	J2	+	+
A1	+	+	J3	+	+
A2	+	+	J4	+	+
A3	-	-	J5	+	+
A4	+	+	J6	-	-
A5	+	+	J7	-	+
A6	+	+	J8	+	+
A7	+	+	J9	+	+
A8	+	+	J10	+	+
A9	-	-	J11	+	+
A10	-	-	J12	+	+
			J13	+	+
			J15	+	+
			J16	+	+
			J17	+	-
			J18	+	+
			J19	+	+
			J20	+	+
			J21	+	+
			J22	+	-

Table 4.2. Ability of the Cdc1 "J" and "A" mutants to rescue *cdc1-P13* a *cdc1* t.s. strain at the restrictive temperature (36.5 °C). 3x is the empty vector, Cdc1 is the positive control. "+" indicates rescue "-" indicates inability to rescue.

From the results above it appears that the majority of mutants behave similarly in their ability to rescue *cdc1Δ* and *cdc1* t.s. In the A mutants the only noticeable difference is the fact that A6 cannot rescue *cdc1Δ* when the promoter is repressed but it can rescue *cdc1* t.s. under the same expression level. This is also found in some of the J mutants (J3, J4, J5, J10, J11, J13 and J21). J16 is not able to rescue the *cdc1Δ* at any protein level but it is able to rescue the *cdc1* t.s. at any level of protein produced. J6 can rescue the *cdc1Δ* when over-expressed, but it cannot rescue the *cdc1* t.s. under any protein level produced. J7 is the only mutant that can only rescue when the promoter is repressed, whereas when the promoter is derepressed it cannot rescue.

This suggests that a high level of the protein is having a deleterious effect whereas a lower level is not and thus it indicates that J7 is behaving as a dominant negative.

4.2.4 A10 and J7 are Dominant Negative in a t.s. Background

In the previous section it was thought that J7 could be acting as a dominant negative. Also when the A mutants were investigated for their ability to rescue the *cdc1-P13* strain the following observation was made. Investigation of the transformants at the permissive temperature (28°C) showed that in some cases growth was better when the promoter was repressed that when it was derepressed. This is indicative of a dominant negative effect and was observed with mutants A3, A8, A9 and A10 (see Table 8.11, appendix A). To test this, mutants A3, A8, A9, A10, J7 and A1 (included as a negative control, as it does not behave as a dominant negative) were again transformed into *cdc1-P13*. They were allowed to grow and then patched on media containing thiamine (promoter repressed) at the permissive temperature (28°C). The patches were then replica plated onto plates with and without thiamine (promoter repressed and derepressed, respectively). To ensure that no thiamine was carried over from the replica plating, the plates were replica plated a second time onto the same medium. Results are shown in Table 4.3, below.

Investigation of the putative dominant negative effect of some Cdc1 mutants on a t.s. background at the permissive temperature (28 °C)		
Mutant	Promoter derepressed	Promoter repressed
A1	+	+
A3	+	+
A8	+	+
A9	+	+
A10	-	+
J7	-	+

Table 4.3. Investigation of the putative dominant negative effect of some Cdc1 mutants on a t.s. background at the permissive temperature (28 °C). + indicates ability to grow, - indicates inability to grow.

It appears from the results that only J7 and A10 are dominant negative on the *cdc1-P13* background. A10 and J7 were transformed into *cdc1-223*, another *cdc1* t.s. strain, to investigate if they continued to exert a dominant negative effect. It was found that they did, see Figure 4.6.

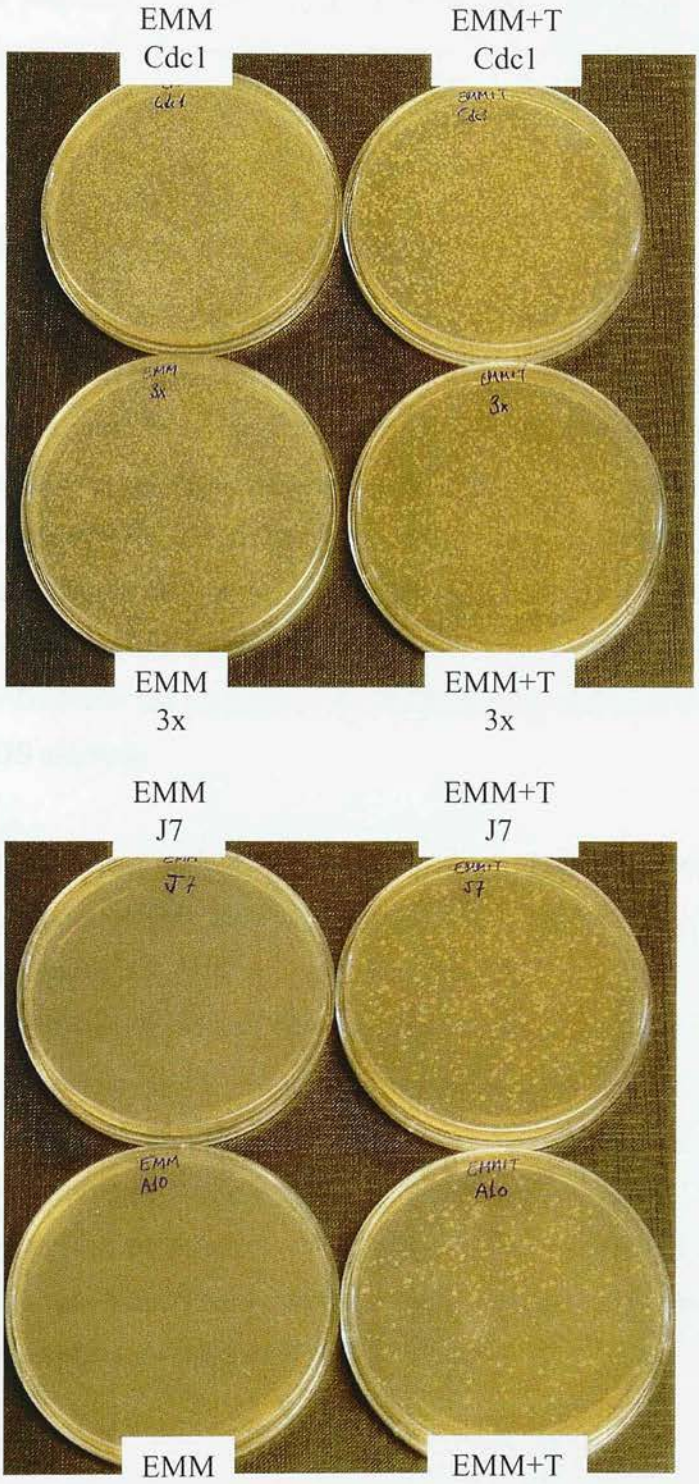

Figure 4.6. Photograph of dominant negative mutants J7 and A10 transformed into *cdc1-223*. Plates were grown at permissive temperature, 28°C, for 4-6 days. EMM plates have promoter derepressed, EMM+T plates have promoter repressed. 3x is empty vector, Cdc1 is wild type control.

The dominant negative effect of J7 and A10 can be clearly seen as colonies can only grow if the promoter of the plasmid carrying these mutations is repressed. The expression level of the controls (Cdc1 and empty vector, 3x) did not affect growth, as expected.

4.2.5 Protein levels of the mutants

The ability of the Cdc1 mutants to rescue both *cdc1Δ* and *cdc1* t.s. strains is described in the previous sections. However, it is possible that the mutants which do not rescue are unable to do so because they do not produce any protein, or this protein is quickly degraded, rather than because the mutation is having any effect. In order to investigate this, the protein levels of the mutant proteins were determined. This was made easy by the choice of plasmid into which the mutants were subcloned, pREP3xH_6BN, which has both an MRGS tag and a hexahistidine tag, which are both N- terminal. To detect the protein levels the MRGS tag was used in conjunction with an anti-MRGS antibody.

The mutants that rescue must have produced protein, so their protein levels were not investigated. Those which were investigated were the following: all the mutants which do not rescue (J1, J16, J7, A3, A9, A10, E2); J17, as it has a stop codon inserted; J6, a mutant that is only able to rescue *cdc1Δ* when overexpressed and J8, a mutant that is able to rescue under any protein expression level, were included as controls, as were wild type Cdc1 and the empty vector. These were all transformed into a haploid wild-type *S. pombe* strain (ED1090; see section 6.1.8), colonies were allowed to grow and total protein was extracted (see section 6.2.1.24). A Western blot using the anti-MRGS antibody was then performed as described in section 6.2.1.25. The results are shown in Figure 4.7, below.

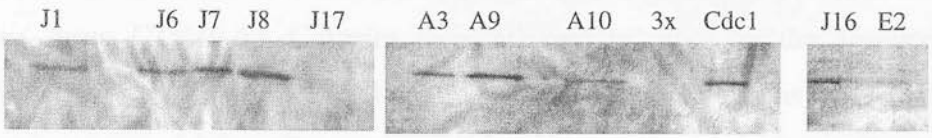

Figure 4.7. Western done with anti-MRGS antibody on a number of *cdc1* mutants. All mutants were in pREP3xH_6BN. 3x is empty vector, Cdc1 is wild type Cdc1.

The results show that all the mutants, with the exception of E2 and J17, have produced protein with an apparent size similar to that of wild type Cdc1. The controls show that J8 and J6 do have protein produced; the empty vector does not have any protein produced; and Cdc1 has protein produced to a similar level to the other mutants. In both E2 and J17 the result of the mutagenesis is the introduction of a stop codon (in E2, this is due to a frameshift). Both mutants had no detectable protein, of any size.

4.2.6 Binding assays with the mutants

In chapter 3 the interaction of Pol3 with Cdc1 was narrowed down to the C- terminus of Pol3 (ZnF2) interacting with Cdc1, but as mentioned above, little is known about the other interactions within the complex. In this section, the interactions between Cdc1 and Pol3, and Cdc1 and Cdc27 were further investigated by two-hybrid. All the mutants were subcloned from pREP3xH_6BN into pBTM116, (see section 6.2.2.8.10) so all the mutants will be produced as LexABD-tagged proteins. The binding assays of Gal4AD-Pol3 to LexABD-Cdc1 were shown, in the previous chapter, to require the presence of myc-tagged Cdc27, therefore, the assays were performed using LexABD-Cdc1 with Gal4AD-Cdc27 and with Gal4AD-Pol3 in the presence of myc-tagged Cdc27.

4.2.6.1 Interactions between Pol3 and Cdc1

This set of liquid culture β- Gal assays were performed with the LexABD-Cdc1 mutants and Gal4AD-Pol3 in the presence of myc-tagged Cdc27. For this pACT-

Pol3, pBTM116-Cdc1 mutants and pAA-Cdc27 were co-transformed into the *S. cerevisiae* strain CTY10-5d. Colonies were allowed to grow on the appropriate media, then they were grown in liquid culture and the β- Gal assay was performed as described in section 6.2.2.5. The binding of LexABD-Cdc1 wild type to Gal4AD-Pol3 was considered 100% and the other mutants were compared to this level of binding. See Figure 4.8, below (Table 8.12 in appendix A shows the raw data for Figure 4.8).

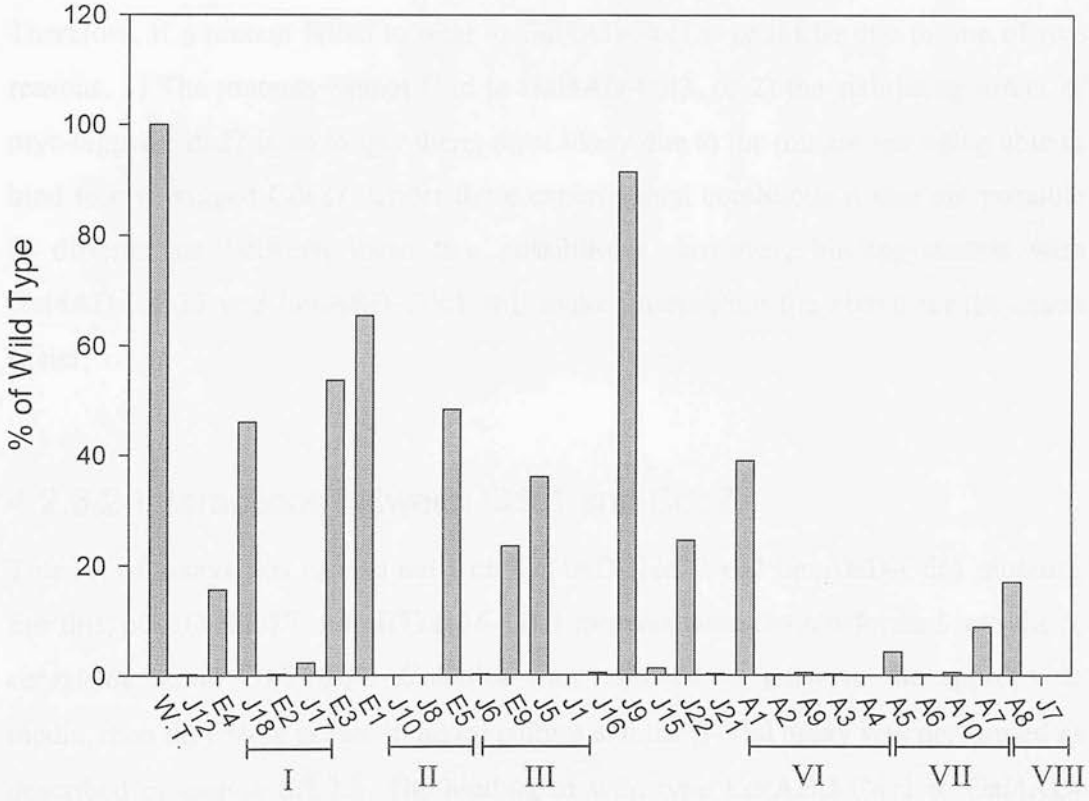

Figure 4.8. Binding assay performed with Gal4AD-Pol3 and LexABD-Cdc1 mutants in the presence of myc-tagged Cdc27. Results are the binding of the mutants compared to wild type, where wild type binding is 100%. The mutants are ordered according to their position within *cdc1*$^{+}$, i.e. those in the N-terminal region are at the left hand side of the diagram etc. Roman numerals indicate conserved boxes identified in a previous study (Reynolds and MacNeill, 1999).

The results of this assay show that some mutants are able to interact with Gal4AD-Pol3 and others are not. It is interesting that there is no clear grouping of mutants that are unable to interact with Gal4AD-Pol3. Such grouping would be indicative of a

region of Cdc1 that will be involved in the binding to Pol3. The closest to what appears to be a grouping of mutants unable to bind to Gal4AD-Pol3 is the region from mutant A2 to A10. This suggests that this region is involved in the binding of LexABD-Cdc1 to Gal4AD-Pol3. However, there are many other mutants that are defective in the binding to Gal4AD-Pol3 (e.g. J12, J16) and there is no clear grouping of them.

It must be remembered however, that these assays were done in the presence of myc-tagged Cdc27, as it is required for the binding of Gal4AD-Pol3 to LexABD-Cdc1. Therefore, if a mutant failed to bind to Gal4AD-Pol3 it could be due to one of two reasons. 1) The mutants cannot bind to Gal4AD-Pol3, or 2) the stabilising effect of myc-tagged Cdc27 is no longer there, most likely due to the mutant not being able to bind to myc-tagged Cdc27. Under these experimental conditions it was not possible to differentiate between these two possibilities, however, binding assays with Gal4AD-Cdc27 and LexABD-Cdc1 will make interpreting the above results much easier.

4.2.6.2 Interactions between Cdc1 and Cdc27

This set of assays was carried out with Gal4AD-Cdc27 and LexABD-Cdc1 mutants. For this, pGAD-Cdc27 and pBTM116-Cdc1 mutants were co-transformed into the *S. cerevisiae* strain CTY10-5d. Colonies were allowed to grow in the appropriate media, then they were grown in liquid culture and the β- Gal assay was performed as described in section 6.2.2.5. The binding of wild type LexABD-Cdc1 to Gal4AD-Cdc27 was considered 100% and the other mutants were compared to this level of binding. See Figure 4.9, below; Table 8.12 in appendix 4 shows the raw data for Figure 4.9.

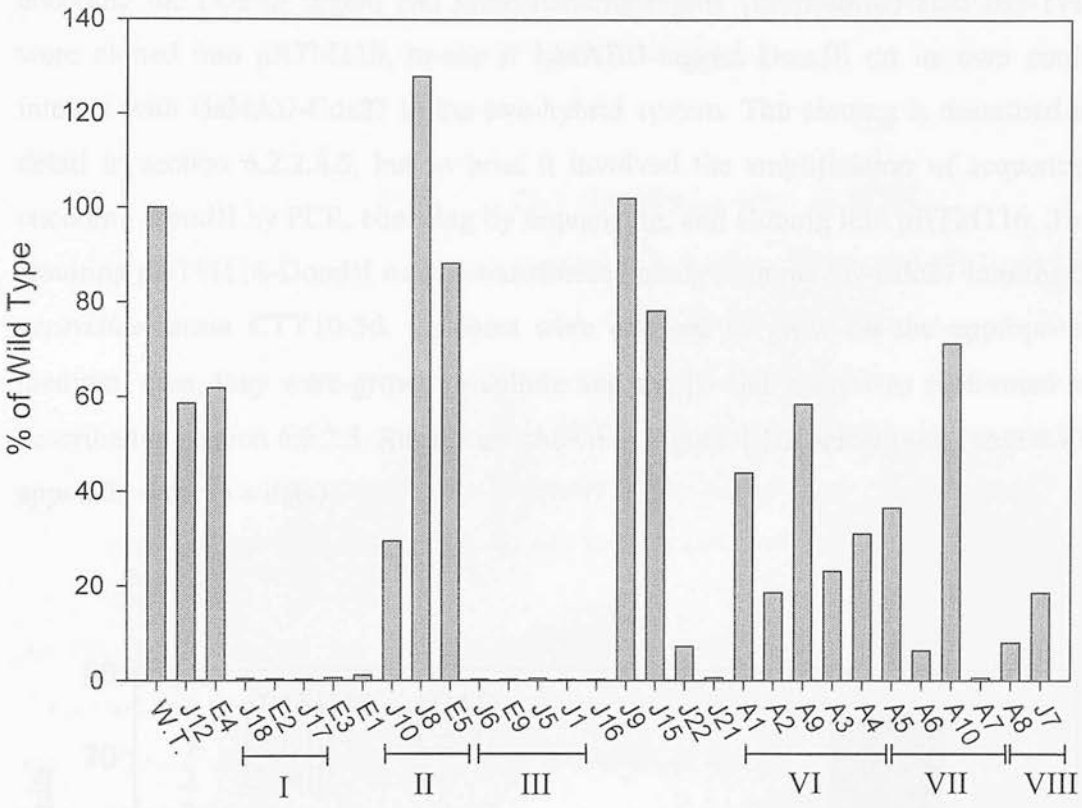

Figure 4.9. Binding assay performed with Gal4AD-Cdc27 and LexABD-Cdc1 mutants. Results are the binding of the mutants compared to wild type, where wild type binding is 100%. The mutants are ordered according to their position within *cdc1+*, i.e. those in the N- terminal region are at the left hand side of the diagram etc. Roman numerals indicate conserved boxes identified in a previous study (Reynolds and MacNeill, 1999).

The results of this set of assays were much clearer. They showed two very clear groupings of mutants that are not able to bind to Gal4AD-Cdc27. These groups are found between mutations J18 to E1 and J6 to J16. Interestingly, one of these regions, the one from J6 to J16 corresponds almost precisely to that of the conserved box III (see Figure 4.4; hereafter conserved box III will be referred to as DomIII).

4.2.6.3 Binding of DomIII to Cdc27

It was shown in section 4.2.6.2 that a grouping of LexABD-tagged Cdc1 mutants unable to bind to Gal4AD-Cdc27 corresponded almost precisely to the conserved region of Cdc1, DomIII. Therefore, it seemed likely that this region of Cdc1 was

involved in the direct binding to Cdc27. To investigate this more fully sequences encoding the DomIII region and some flanking region (from amino acid 108-171) were cloned into pBTM116, to see if LexABD-tagged DomIII on its own could interact with Gal4AD-Cdc27 in the two-hybrid system. The cloning is described in detail in section 6.2.2.8.6, but in brief it involved the amplification of sequences encoding DomIII by PCR, checking by sequencing, and cloning into pBTM116. The resulting pBTM116-DomIII was co-transformed along with pGAD-Cdc27 into the *S. cerevisiae* strain CTY10-5d. Colonies were allowed to grow on the appropriate medium, then, they were grown in culture and the β- Gal assay was performed as described in section 6.2.2.5. Results are shown in Figure 4.10, below (see Table 8.13, appendix 4 for raw data).

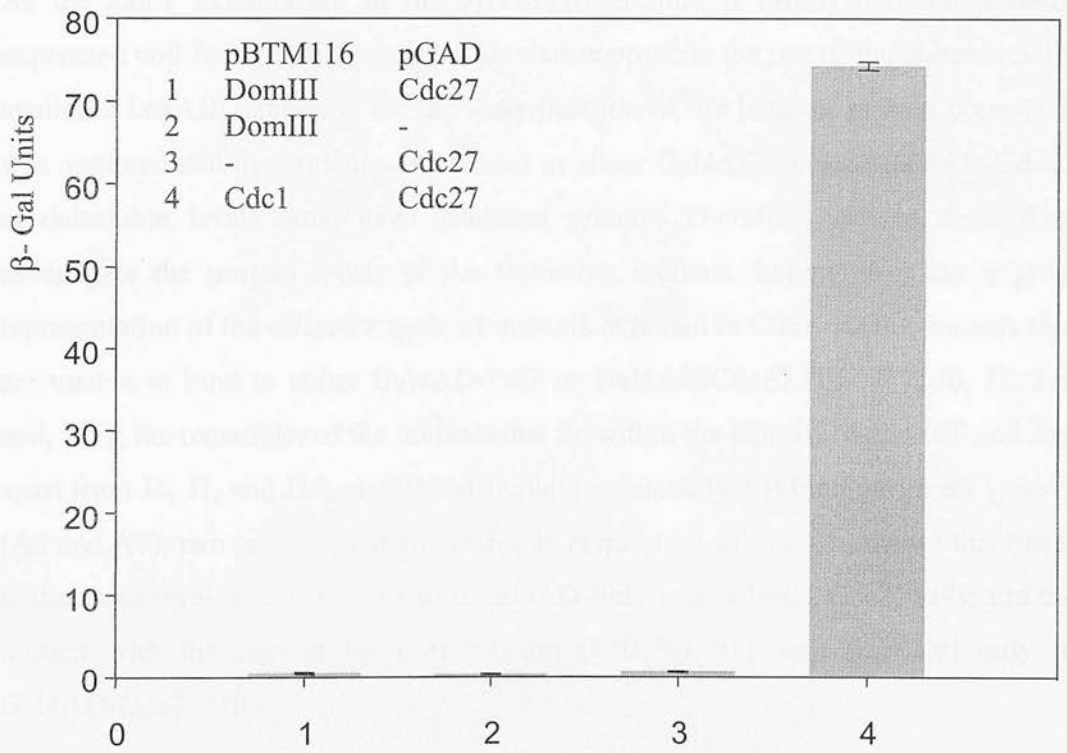

Figure 4.10. Binding assay to test for interaction between LexABD-DomIII and Gal4AD-Cdc27. "-" indicates that the empty vector was used. Assays were done in triplicate, column height is the mean of the values and the error bars are the standard deviation from the mean.

As can be seen from Figure 4.10 above, full length LexABD-Cdc1 is able to bind to Gal4AD-Cdc27, as expected. On the other hand, when LexABD-DomIII alone was tested for binding to Gal4AD-Cdc27 no binding was detected.

4.2.7 Expression levels of Cdc1 mutants and DomIII

Some of the LexABD-Cdc1 mutants were unable to bind to either Gal4AD-Cdc27 or to Gal4AD-Pol3 in the β- Gal assay. This could be because they are not being expressed, as was investigated before (see section 4.2.5) in regard to the rescue of *cdc1Δ*. Because the mutants are now in a different plasmid (pBTM116 as opposed to pREP3xH_6BN) and growing in a different yeast background (*S. cerevisiae* as opposed to *S. pombe*) it was decided to once more check their protein levels.

As the Cdc1 mutants are in the pBTM116 plasmid it means that the proteins expressed will be LexABD-tagged. This makes possible the use of the commercially available LexABD antibody for the determination of the level of protein present. It was assumed that the mutants which bind to either Gal4AD-Pol3 or Gal4AD-Cdc27 at detectable levels must have produced protein. Therefore, it was decided to investigate the protein levels of the following mutants, hoping they are a good representation of the different types of mutants obtained in Cdc1: all the mutants that are unable to bind to either Gal4AD-Pol3 or Gal4AD-Cdc27 (E2, J17, J6, J1, J16 and, J21); the remainder of the mutants that lie within the DomIII region (E9 and J5), apart from J6, J1, and J16, mentioned above; two mutants that bind only very weakly (A6 and A7); two mutants that are unable to rescue (A9 and A3); a mutant that binds to the same level as wild type to both Gal4AD-Pol3 and Gal4AD-Cdc27 (J9); and the mutant with the highest level of binding (>100%), although it bound only to Gal4AD-Cdc27 (J8).

The protein level of LexABD-DomIII was also investigated to see if there was any protein produced and, if so whether it was expressed to the same level as LexABD-Cdc1. To investigate the different constructs as described above they were all transformed individually into the *S. cerevisiae* strain CTY10-5d, colonies were

allowed to grow and total protein extracts were performed on them as described in section 6.2.1.22. 10 μg of total protein was subjected to SDS-PAGE, transformed onto a PVDF membrane and detection was performed with the anti-LexABD antibody as described in section 6.2.1.25. Results are shown in Figure 4.11, below.

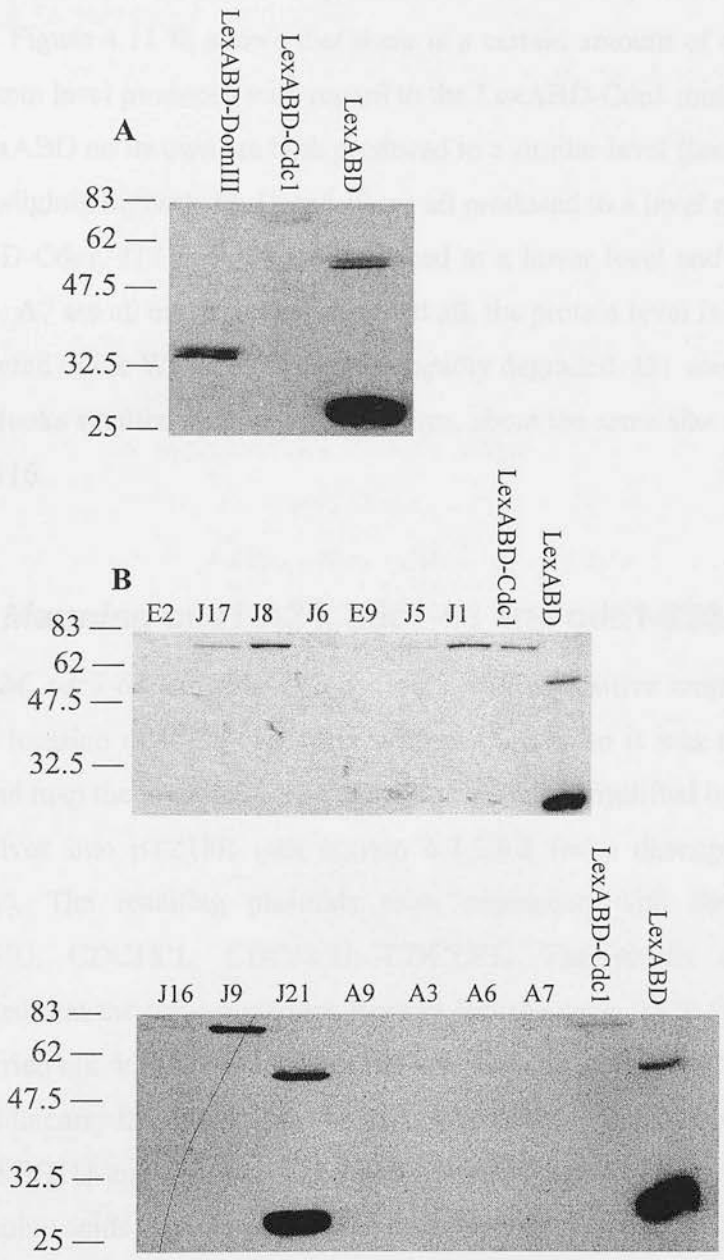

Figure 4.11. Westerns done with the HRP-conjugated anti-LexABD antibody on LexABD-DomIII and some LexABD-Cdc1 mutants. **A**, Western with LexABD-DomIII; **B**, Westerns with LexABD-Cdc1 mutants. Cdc1 is wild type control, LexABD is expressed from the empty vector pBTM116. The mutants are ordered according to their position within *cdc1*$^{+}$, i.e. those in the N- terminal region are at the left hand side of the diagram etc. An estimated 10 μg of total protein was loaded in each lane.

As can be seen from Figure 4.11 A, it appears that LexABD-DomIII and LexABD on it own (expressed from pBTM116) are both present at high levels. On the other hand, LexABD-Cdc1 is present at a lower level. Hence, it seems unlikely that the protein level of LexABD-DomIII is responsible for its inability to interact with Gal4AD-Cdc27. Figure 4.11 B, shows that there is a certain amount of diversity in terms of the protein level produced with regard to the LexABD-Cdc1 mutants. LexABD-Cdc1 and LexABD on its own are both produced to a similar level (level of LexABD on its own is slightly higher). J8, J1 and J9 are all produced to a level comparable to that of LexABD-Cdc1. J17 and J16 are produced to a lower level and E2, J6, E9, J5, A9, A3, A6, A7 are all either not expressed at all, the protein level is so low that it cannot be detected in the Western, or they are rapidly degraded. J21 was not of the expected size, it looks smaller than the other mutants, about the same size as the band found in pBTM116.

4.2.8 Mapping cdc1-A24, cdc1-64 *and* cdc1-223.

cdc1-A24, *cdc1-64* and *cdc1-223* are temperature sensitive mutations of *cdc1*$^{+}$. The precise location of these mutations was not known so it was decided to sequence them and map the mutations. The mutant genes were amplified by PCR and cloned in two halves into pTZ19R (see section 6.2.2.8.2 for a description of the cloning strategy). The resulting plasmids were sequenced with the following oligos: CDC15'U, CDC15'L, CDC13'U, CDC13'L. The results of this sequencing suggested that the three mutations were in fact the same. PCR (see section 6.2.1.15) was carried out with primers CDC13'U and CDC13'L to amplify the region of *cdc1* thought to carry the mutations. The PCR fragments were sequenced with the internal oligos SEQ1U and SEQ2L. The results showed that all three mutants map to the same amino acids Asp389 (GAT) and contained the same mutation, Asn389 (AAT).

4.3 Discussion

The mutational analysis of Cdc1 has proved to be informative in a number of ways. Firstly, when the transposon based random mutagenesis was carried out it became apparent that there were hotspots for mutations in Cdc1 as mutations tended to be localised to particular sites rather than throughout the length of the gene. Even though the mechanism is most likely different, evidence suggesting the existence of mutational hotspots was supported by the fact that when the three independent t.s. mutations described in section 4.2.8 were mapped, the three mapped to the same nucleotide.

The ability (or not) of the mutants to rescue a strain deleted for Cdc1 has shown that although Cdc1 is a highly conserved protein, it is remarkably tolerant to mutations. Of the 30 different mutations that were introduced in Cdc1, 14 were able to rescue at all protein levels, nine were able to rescue only when overexpressed, while seven were unable to rescue at any protein level. Hence, it appears that the introduction of five amino acids (even when falling within a conserved region) or the mutation of certain highly conserved amino acids has little effect on the ability of Cdc1 to rescue. The seven mutants that did not rescue *cdc1Δ* were E2, J1, J16, A9, A3, A10, and J7. Of these E2, as described before, has a sixteen base pair insertion resulting in a frameshift and the introduction of a stop codon. A Western blot of this mutant showed no detectable protein levels present, thereby explaining the inability of this mutant to rescue a *cdc1Δ* strain. Western analysis of the other mutants that were unable to rescue showed that they do produce protein, hence, their inability to rescue is not due to a lack of protein as in the case of E2. It is interesting to note that two of the three pentapeptide insertion mutants (J1 and J16) lie very close together. J1 lies in a region of Cdc1 that is highly conserved, conserved box III (DomIII) and J16 lies very close to that region. Furthermore, all of the other mutants that fall in DomIII (J6, J4 and E9) are only able to rescue if they are overexpressed. The fact that no other such grouping of mutants is seen, with most of the mutants that fall within the other conserved regions being able to rescue suggests that DomIII is an extremely important region, and may be involved in the function of Cdc1.

The results obtained with mutant J17 were intriguing. This mutant has a stop codon inserted at amino acid 28 and Western analysis failed to detect any protein being produced. Therefore, it was expected that this mutant would not rescue the deletion strain. However, when the experiment was carried out it was found that the mutant, when overexpressed, did indeed rescue the deletion strain. This was very surprising, especially since a deletion of the first 50 amino acids of Cdc1 prevents the rescue of a *cdc1Δ* strain (MacNeill *et al.,* 1996). Three possibilities could account for the rescue by J17 a) the mutant has a stop codon but the existence of a nearby start codon allows for the ribosome to re-initiate translation and complete the protein, b) the presence of a start codon after the stop codon can act as an internal ribosome entry site (reviewed in Hellen and Sarnow, 2001) and a slightly truncated protein is produced or c) the stop codon is ignored by the translation machinery resulting in a low level of full length Cdc1 being produced. The nearest start codon after amino acid 28 is located at amino acid 78, which would seem to rule out the first hypothesis as the ribosome could only re-start translation without dissociation if the start codon was close to the stop codon. The second and third hypothesis are possible although the third hypothesis seems like the most likely, given the fact that J17 is only able to rescue when overexpressed. When the protein is overexpressed there will be more instances of the translation machinery ignoring the stop codon and hence, there will be a higher level of full length protein being produced. If this is the case then J17 will be of the same size as the full length protein. It was hoped that the H_6 tag, in combination with Ni- agarose, could be used to purify this mutant protein and investigate whether its size is similar to that of wild type Cdc1, and hence that the stop codon is being ignored, or whether there is a size difference and a truncated form of the protein is being produced. However, attempts to purify wild type Cdc1 by this method were unsuccessful, and therefore, it proved impossible to do this experiment. Nonetheless, it is seems likely that the hypothesis that the stop codon is being ignored to produce low levels of full length protein is the correct one.

It was very surprising that most of the site directed mutants were able to rescue, since the mutations all involved conserved amino acids. It was expected that these conserved amino acids would have an important role and therefore their mutation

would result in an inability to rescue the *cdc1Δ* strain. The fact that these mutants are able to rescue a *cdc1Δ* strain suggests that the role of the conserved amino acids, if any, is not affected by mutating them, or that a mutation to alanine does not have much of an effect. In the two instances where two amino acids are conserved and consecutive (as in mutants A1 and A2, and A5 and A6) the single mutants are viable but the double mutant is not. This could mean that redundancy exists; if one amino acid is mutated then the other can still either maintain the structure of Cdc1 or perform its function, but if both are mutated then the mutant is not able to rescue. The single amino acid mutated in A3 is most likely a very important amino acid as its mutation from aspartic acid to alanine is enough to abolish rescue of the deletion strain. Hence, it appears, that whereas most of the site directed mutants are able to rescue, A3 and the two double mutants are not, suggesting that these amino acids are very important for Cdc1.

The ability of the mutants to rescue a *cdc1* t.s. strain was investigated and it was found to be similar to the ability of the mutants to rescue a *cdc1Δ* strain. Most mutants that are able to rescue a *cdc1Δ* are also able to rescue a *cdc1* t.s. However, in general the mutants were able to rescue the t.s. strain slightly more efficiently than the *cdc1Δ*. The only exception was J6 which appears to be unable to rescue the t.s. strain at any protein level, but which is able to rescue the deletion strain when overexpressed. It was also discovered that in a t.s. background A10 and J7 behave as dominant negatives. The fact that these two mutants are also unable to rescue under any protein level produced suggests that the region in which they fall might have an important role in Cdc1.

The binding assays (LexABD-Cdc1 mutants with Gal4AD-Pol3 in the presence of myc-tagged Cdc27, and LexABD-Cdc1 mutants with Gal4AD-Cdc27) are compared in Figure 4.12, below, which also shows the ability of the mutants to rescue a Cdc1 deletion strain.

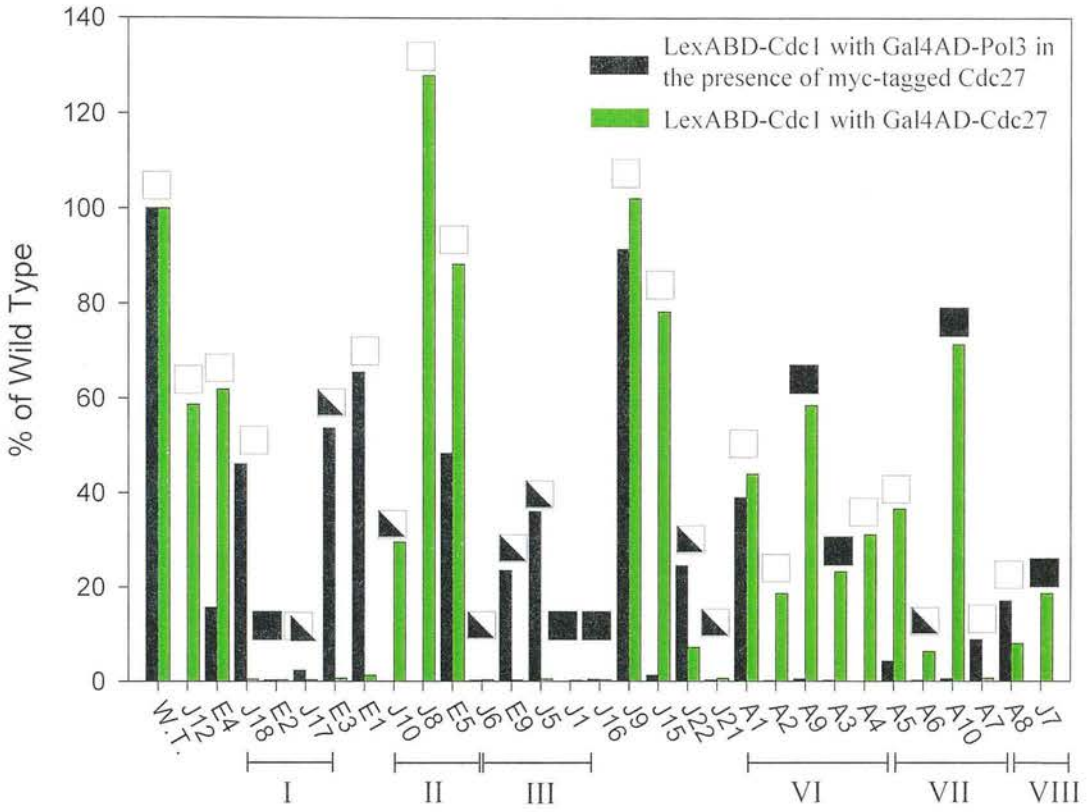

Figure 4.12. Graph comparing binding assays of LexABD-Cdc1 mutations with Gal4AD-Cdc27 (light green) and LexABD-Cdc1 mutations with Gal4AD-Pol3 in the presence of myc-tagged Cdc27 (dark green). Results are compared to wild type where wild type binding is 100%. Filled boxes indicate that the mutant is unable to rescue a deletion strain under any protein level produced; white boxes indicate that the mutant is able to rescue at all protein levels and half-filled boxes indicate that the mutant is only able to rescue when produced to high level. The mutants are ordered according to their position within *cdc1+*, i.e. those in the N- terminal region are at the left hand side of the diagram etc. Roman numerals indicate conserved boxes identified in a previous study (Reynolds and MacNeill, 1999). Figure is a combination of Figure 4.5, Figure 4.8, and Figure 4.9.

Figure 4.12, above, shows that there is no simple correlation between the ability of a given mutant to rescue a *cdc1Δ* strain and the ability to bind to the components of polδ. In the study of the interactions between Gal4AD-Pol3 and LexABD-Cdc1 mutants, there is no instance of a mutant that is unable to rescue but that is able to bind to Gal4AD-Pol3, which suggests that the inability to rescue *cdc1Δ* and the inability to bind to Gal4AD-Pol3 go hand in hand. There appears to be no other correlation between ability to rescue *cdc1Δ* and the levels of binding to Gal4AD-Pol3. Interestingly however, in the case of LexABD-Cdc1/Gal4AD-Cdc27

interactions, there are cases in which a mutant is able to bind to Gal4AD-Cdc27 but is not able to rescue the *cdc1Δ* strain under any protein level produced (A9, A3, A10 and to a lesser degree J7). This implies that binding of Gal4AD-Cdc27 to LexABD-Cdc1 is independent of the ability of the mutant to rescue. On the other hand, as stated above, the inability to rescue a deletion strain appears to indicate that Gal4AD-Pol3 will not be able to bind to that mutant.

As mentioned above, Gal4AD-Pol3 cannot bind to LexABD-Cdc1 if myc-tagged Cdc27 is not present. Therefore, in the study of the interactions between Gal4AD-Pol3 and LexABD-Cdc1, the failure of LexABD-Cdc1 mutants to bind to Gal4AD-Pol3 could be due to that mutant having lost the stabilising effect of myc-tagged Cdc27, most probably due to the affected LexABD-Cdc1 mutant being unable to bind to it. Thus, LexABD-mutants that appear not to bind to Gal4AD-Pol3 could be misleading. For example, mutant J6 cannot bind to Gal4AD-Cd27 and this may be the cause of its apparent inability to bind to Gal4AD-Pol3. Other mutants, unable to bind to Gal4AD-Pol3 but which can bind to Gal4AD-Cdc27 will therefore, retain the stabilising effect of myc-tagged Cdc27. For example, A9 cannot bind to Gal4AD-Pol3, but can to Gal4AD-Cdc27 suggesting that it is indeed defective in binding to Gal4AD-Pol3. Unfortunately, it will not be possible to distinguish between mutants that are unable to bind to Gal4AD-Cdc27 and mutants that are unable to bind both to Gal4AD-Pol3 and Gal4AD-Cdc27. It was expected that all the mutants that were unable to bind to Gal4AD-Cdc27 would be unable to bind to Gal4AD-Pol3, due to the loss of the stabilising effect of myc-tagged Cdc27. However, there were five examples (E3, E1, E9, J5 and to a lesser degree A7) of mutants that were not able to bind to Gal4AD-Cdc27 but were able to bind to Gal4AD-Pol3 in the presence of myc-tagged Cdc27. The reason for this is not entirely clear but it suggests that in some cases Gal4AD-Pol3 could have a stabilising effect on the interaction between myc-tagged Cdc27 and LexABD-Cdc1.

After analysis of the binding assays, regions thought to be important for the interactions between LexABD-Cdc1, Gal4AD-Pol3 and Gal4AD-Cdc27 have been identified. The Cdc1 region required to bind to Gal4AD-Pol3 appears to run from

amino acid 293 to amino acid 329 as mutants within this region (A2 to A10) are able to bind to Gal4AD-Cdc27 but are not able to bind to Gal4AD-Pol3. This includes conserved boxes VI and VII of Cdc1. This is the only clear grouping of this type to be seen in the binding assays with Gal4AD-Pol3. Interestingly, this region contains mutant A3, the single amino acid mutant that is unable to rescue *cdc1Δ*, suggesting that the role of the amino acid mutated in A3 could be involved in the binding to Pol3. Further, this region contains A10, which is one of the mutants that was found to have a dominant negative effect.

The region of LexABD-Cdc1 involved in binding to Gal4AD-Cdc27 is less clear as two groupings were found: one formed by the mutants J18, E2, J17, E3 and E1; the other formed by the mutants J6, E9, J5, J1 and J16. Of these two regions the second one is the most interesting as it corresponds almost precisely to DomIII. DomIII, as mentioned before, is interesting because two of the three pentapeptide insertion mutants that are unable to rescue are located in it or very close to it. Further, the other mutants that fall in this region will only rescue when over-expressed. These results suggest that this region is involved in binding to Gal4AD-Cdc27. The other region, comprised of mutations J18, E2, J17, E3 and E1, is not as interesting. E2 and J17 both contain a stop codon and Western analysis (see Figure 4.11) has shown that E2 is not expressed and J17 is expressed at a reduced level which could explain their lack of binding to both Gal4AD-Pol3 and Gal4AD-Cdc27. Also, J17 is only able to rescue when over expressed. J18 is able to rescue at all levels of protein produced, therefore, this does not point to this area of the region being very important. The remainder of the region (mutants E3 and E1), is not as convincing as DomIII as it does not fall within a conserved region of Cdc1. Furthermore, E1 is able to rescue at all levels of protein produced. Therefore, it was thought that DomIII is involved in the binding to Gal4AD-Cdc27.

This hypothesis however, appears to contradict previous results by E. Murray-Smith in the lab. A two-hybrid screen was carried out with Cdc27 as bait and the interacting plasmid proved to contain truncated Cdc1 that began at amino acid 157, which lies downstream of DomIII. The difference between this two-hybrid result and the one

obtained in this study could be due to the fact that in the previous two-hybrid screen a different plasmid was used (pGBT9). To elucidate this further, sequences encoding DomIII were cloned into pBTM116 to investigate its direct interaction with Gal4AD-Cdc27 (as described in section 4.2.6.3). When the binding assay was performed it was found that whereas LexABD-Cdc1 was able to bind to Gal4AD-Cdc27, LexABD-DomIII was not able to do so. Western analysis performed on LexABD-DomIII showed that protein was produced, to a level that was similar to that of LexABD on its own, (produced from pBTM116) and apparently to a higher level than LexABD-Cdc1, the wild type control. Therefore, the inability of LexABD-DomIII to bind to Gal4AD-Cdc27 appears not to be due to insufficient protein being present. The Western analysis of some of the LexABD-Cdc1 mutants shed more light on DomIII. It appears that of the five mutants within DomIII only one of them (J1) has a protein level that is detectable on a Western. Hence, it appears that all of the mutants that fall in DomIII (except J1) are not able to interact with Gal4AD-Cdc27 because they do not have protein present to a level that is high enough. Therefore, it appears that LexABD-DomIII is not involved in the direct binding to Gal4AD-Cdc27. It is likely however, that DomIII is important for Cdc1, probably for the function of Cdc1.

As mentioned above, in some cases (E3, E1, E9, J5 and to a lesser degree A7) Gal4AD-Pol3 appears to exhibit a stabilising effect on the interaction of LexABD-Cdc1 to myc-tagged Cdc27. Two of the mutants that show this effect, E9 and J5 do not produce any detectable level of protein by Western analysis. The reason for the stabilising effect of Gal4AD-Pol3 is unclear, but a possible explanation could be as follows. The LexABD-Cdc1 mutants mentioned have the ability to bind to both Gal4AD-Pol3 and Gal4AD-Cdc27, although the interaction with Gal4AD-Cdc27 is so weak as to not produce detectable β- Gal activity. This very weak binding to Gal4AD-tagged or myc-tagged Cdc27 could be enough to stabilise the LexABD-Cdc1 mutant/Gal4AD-Pol3 interaction enabling it to produce a detectable level of binding. Hence, it might not be a stabilising effect of Gal4AD-Pol3, but very weak binding with Gal4AD-tagged or myc-tagged Cdc27.

Western analysis shows that mutant E2 does not produce protein at any detectable level. This was as expected because E2 contains a sixteen base pair insertion that introduces an in frame stop codon. However, J17 which contains a stop codon located at amino acid 28, yields more unusual results (see above). In a Western it can be seen that J17 yields a protein product of the same size as wild type LexABD-Cdc1 albeit at a lower level than LexABD-Cdc1. This implies that the protein is not truncated but rather that the stop codon is being over-ridden at a low level. When a Western was carried out with J17 in a different plasmid (pREP3xH_6BN), in *S. pombe*, using a different antibody (anti-MRGS), it failed to detect any J17 being expressed. Hence, it appears that either the expression level is affected by the choice of plasmid, or the anti-Gal4AD antibody was more sensitive or the stop codon is ignored in *S. cerevisiae* more frequently. Nonetheless, it appears that the reason for J17 rescuing the *cdc1Δ* and the t.s. when overexpressed is due to the stop codon being ignored, producing a low level of full length protein.

The mutants that are able to bind to Gal4AD-Cdc27 very well (J8 and J9) both have protein levels that are very high. Two mutants that are able to bind very weakly do not have any detectable protein level, which is most likely the reason for their weak level of binding. The two mutants that are unable to rescue but are able to bind to Gal4AD-Cdc27 (A9, ~58% and A3, ~23%) do not appear to have any detectable protein level. This was unexpected but it could mean that in this region the protein level does not need to be very high for the binding to Gal4AD-Cdc27.

In summary, in this chapter the extensive mutational analysis of Cdc1 has discovered that the protein contains areas that are hotspots for mutations and that Cdc1 is very tolerant to mutations, both point mutations and five amino acid insertions. Further, investigations into whether the mutants can rescue *cdc1Δ* have identified regions that are thought to be important for Cdc1 structure and/or function. Two-hybrid analysis has shown that a region of LexABD-Cdc1 thought to be important for binding to Gal4AD-Cdc27 (DomIII) is not involved in the direct binding, and a region of LexABD-Cdc1 thought to be involved in the binding to Gal4AD-Pol3 (from A2 to A10) has been identified.

5 Discussion

5.1 Summary of the work presented in this thesis

In this thesis the interactions within DNA polymerase δ in *S. cerevisiae* and *S. pombe* have been investigated. In *S. cerevisiae* the region of the catalytic subunit (Pol3) involved in the binding to the B subunit (Pol31) was narrowed down to the C-terminus of Pol3, specifically to the last putative C- terminal zinc finger, ZnF2, using a two-hybrid assay. Further, mutational analysis of ZnF2 discovered amino acids that are important for the binding to Pol31. All four cysteines in the ZnF2 are required for binding but the histidine in the zinc finger is not required, also a number of other mutations in ZnF2 diminished the binding to Pol31. This suggests that these amino acids are important for the ZnF2 structure. Interestingly, the Pol31 mutant Sdp5 (K358E) is able to improve the binding of most of these ZnF2 Pol3 mutants. This suggests that the region mutated in Sdp5 is involved in the binding to Pol3. Further, using a two-hybrid binding assay it was shown that the ZnF2 region is not only sufficient but necessary for binding to Pol31.

In *S. pombe* the region of Pol3 involved in the binding to the B subunit (Cdc1) was narrowed down by two-hybrid analysis to the C- terminus of Pol3, in particular the region that consists exclusively of the two C- terminal zinc fingers. In the two-hybrid system this interaction was shown to be dependent on the presence of the C subunit (Cdc27). Using the two-hybrid system it was not possible to show any interaction between ZnF2 alone and Cdc1, although a colleague in the lab, Dr. Leo Ciufo, has demonstrated *in vitro* that the Pol3 ZnF2 and Cdc1 are able to interact. Mutational analysis has shown that, just as in *S. cerevisiae*, the four cysteines in *S. pombe* Pol3 ZnF2 are involved in the binding to Cdc1. As mentioned above, the two-hybrid interaction between Pol3 and Cdc1 required the presence of Cdc27. It was shown that Cdm1 (the D subunit in *S. pombe* polδ) was not able to substitute for the stabilising effect seen with Cdc27 on the interaction between Pol3 and Cdc1.

The B subunit of *S. pombe* polδ, Cdc1, was subjected to both random and site directed mutagenesis. These mutations were investigated for their ability to rescue a *cdc1Δ* and a *cdc1* t.s. strain. The results revealed a region of Cdc1 that is important for its function (DomIII). These mutants were then used in two-hybrid binding assays to try and narrow down the regions of Cdc1 that might be involved in the binding to Pol3 and Cdc27. This approach has highlighted a region of Cdc1 that may be involved in the binding to Pol3 as the Cdc1 mutants that lie within this region are unable to bind to Pol3.

5.2 Regions of the A subunit involved in binding to the B subunit

The data obtained in this thesis with regard to the binding of the A and B subunits has come from both *S. cerevisiae* and *S. pombe*. The investigation was guided by the limited amount of data that suggested that the A subunit interacts with the B subunit via the C- terminal domain of the A subunit. This data was obtained in mouse polα (Mizuno *et al.,* 1999) and *S. cerevisiae* polδ (Giot *et al.,* 1995) and polε ((Dua *et al.,* 2000); see section 1.3.3.2.1). The data obtained in this thesis from both *S. pombe* and *S. cerevisiae* involves the second zinc finger (ZnF2) of the A subunit in the direct binding to the B subunit. There is no discrepancy in the data obtained from *S. cerevisiae* and *S. pombe*. This data is also in accordance with the data mentioned above from mouse polα and *S. cerevisiae* polε. Further, as both the catalytic subunits of polα and ε have C- terminal zinc fingers it is tempting to speculate that the function of the zinc finger region in the A subunit of polα, δ and ε is to interact directly with the B subunit.

Zinc fingers are generally thought of as being involved in binding nucleic acids and not other proteins as shown above. Zinc fingers were first discovered in the transcription factor IIIA of *Xenopus* (Klug and Rhodes, 1987) in which tandem repeats of amino acids were found, which contained a stretch of amino acids with CCHH sequence. Similar repeats were then found in other DNA binding proteins.

These repeats were later found to be individual domains folded around a single zinc ion. Now it is estimated that proteins that contain zinc fingers are one of the most abundant types of proteins in eukaryotes (Laity *et al.*, 2001). It was discovered that the zinc finger domains were able to bind to DNA and RNA and this was thought to be their sole function (Klug and Rhodes, 1987). However, it has been shown that they are also able to mediate protein-protein interactions (MacKay and Crossley, 1998). Some proteins for example Ikaros, a protein involved in lymphoid cell development, even have separate zinc fingers with distinct capabilities to bind DNA and protein.

Zinc fingers are classified depending on the amino acids involved in the binding and the spacing between these residues. The classical zinc finger is called the CCHH finger (which indicates the residues involved in co-ordinating the zinc) although another nine types have been identified. These range from relatively simple domains like the CCHC from the nucleocapsid or MetRS proteins, in which one zinc ion is co-ordinated by the CCHC, to the more complex RING finger or PKC CRD proteins, in which two zinc ions are co-ordinated by a combination of a total of eight cysteines or histidines (Schwabe and Klug, 1994).

The highly conserved ZnF2 sequence of the catalytic domain (Pol3) in polδ is shown in Figure 5.1 below.

```
Sc RLWTQCQRCAGNLHSEVLCSNKNCDIF
Sp RLWTQCQRCQGSMHQDVICTSRDCPIF
Hs RLWTQCQRCQGSLHEDVICTSRDCPIF
Dm RLWTECQRCQESLHEEVICSNRDCPIF
Mm RLWTQCQRCQGSLHEDVICTSRDCPIF
     C  C        C    C
```

Figure 5.1. Sequence of ZnF2 from Pol3 of polδ. Amino acids underlined in the *Sc* sequence indicate the location of two t.s. mutations. *Sc, S. cerevisiae; Sp, S. pombe; Hs, H. sapiens; Dm, D. melanogaster; Mm, M. musculus.*

It was shown in chapter 2 that the histidine appears not to be involved in binding the zinc, but that the four cysteines are. Therefore, ZnF2 appears to be C-X_2-C-X_9-C-X_4-C. One of the common features of zinc fingers is that they all appear to have a common motif called a zinc knuckle (Schwabe and Klug, 1994) which has the conserved C-X_2-C sequence. This motif is present in ZnF2 as can be seen in Figure 5.1. The ZnF2 structure as a whole appears to be similar to some types of zinc finger although it appears not to fit in perfectly with the amino acids and spacing of any of the zinc fingers described by Schwabe and Klug (1994) or by MacKay and Crossley (1998). ZnF2 appears to be similar to the zinc fingers found in the human A20 F7 protein, which have the following sequence: C-X_4-C-X_{11}-C-X_2-C (MacKay and Crossley, 1998), in GATA-1: C-X_2-C-X_{17}-C-X_2-C and the second zinc finger of hormone (oestrogen) receptor: C-X_5-C-X_9-C-X_2-C (Schwabe and Klug, 1994). GATA1 and hormone (oestrogen) receptor have both been shown to interact with DNA (Schwabe *et al.,* 1993; Omichinski *et al.,* 1993). A20 F7 is a tumour necrosis factor inducible protein and it contains seven zinc fingers in its C- terminus. It has been shown that this zinc finger rich domain is involved in protein-protein interactions both with itself and with other proteins (deValck *et al.,* 1996). Therefore, ZnF2 is probably a similar type of zinc finger as the ones in A20 F7, given that they are both involved in protein-protein interactions and both are of the CCCC type with similar spacing.

5.3 Regions of the B subunit involved in binding to the A subunit

An *S. cerevisiae* Pol31 mutant, Sdp5, has been shown to have a positive effect on the binding to Pol3 compared to w.t. Pol31, thereby, implicating this mutated region of Sdp5 in the binding to Pol3. The Sdp5 mutation is a K358E mutation (Giot *et al.,* 1997). From the sequence alignment in Figure 1.3 it can be seen that the site of the Sdp5 mutation is in the conserved domain VII of the B subunit (Pol31). According to the screen performed in chapter 4, the region of Cdc1 thought to be involved in binding to Pol3 was between amino acids 293 and 329, as the mutants located within this region (mutants A2 to A10) fail to bind to Pol3 (although they are capable of

binding to Cdc27). The *S. pombe* homologue of the Sdp5 mutation corresponds to one amino acid after the A7 mutation (G344A) i.e. outwith the putative Pol3 binding region. The ending of the region before mutant A7 is due to the fact that there is a slight level of binding between mutant A7 and Pol3. However, as this binding is only to ~9% of w.t. it is not considered as very significant binding. Thus, it is plausible that the region of Cdc1 involved in the binding to Pol3 is larger and includes this A7 mutant. Hence, it appears that the data between *S. cerevisiae* and *S. pombe* agree with each other and that the region of the B subunit important for the binding to Pol3 lies between amino acids 293 and 345 in *S. pombe*, amino acids 301 to 358 in *S. cerevisiae*. However, the data in chapter 3 shows that Cdc1Δ453 is unable to bind to Pol3 in the two-hybrid system, which suggests that this region is involved in the binding to Pol3, contradicting the before mentioned hypothesis. Further, this truncated mutant is able to bind to Cdc27 in the two-hybrid system (MacNeill *et al.*, 1996), thereby indicating that the lack of binding to Pol3 is not due to the loss of the stabilisory effect seen with Cdc27 (see chapter 3). However, according to the sequence alignment shown in Figure 1.3 there is no homology between the last ten amino acids of Cdc1 and the other B subunits. Further, some B subunits have shorter sequences that stop before the last amino acids of Cdc1 are reached. Therefore, the region between amino acids 293 and 345 is the region most likely to be involved in the binding to Pol3. For ease of reference this region of the B subunit will hereafter be called the Pol3 binding region.

There are two point mutations in *cdc1* that can be rescued by slight overproduction of Pol3. These mutants are *cdc1-18* and *cdc1-M78* and are located at amino acids 293 and 381 respectively (MacNeill *et al.*, 1996). Interestingly, *cdc1-18* lies within the Pol3 binding region, it is in fact located at the same position as the site directed mutant A2 (described in chapter 4). *cdc1-18* is a G to S mutation whereas A2 is a G to A mutation. Therefore, the most likely reason for the t.s. phenotype of *cdc1-18* is that this mutant, similarly to A2, has reduced binding to Pol3. This could also explain the rescue observed with the slight overexpression of Pol3 (MacNeill *et al.*, 1996). *cdc1-M78* is located slightly away from the Pol3 binding region (at amino acid 381). Further, located at amino acid 374 is site directed mutant A8, which can bind to Pol3

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to ~17% (w.t. binding is 100%). This seems to suggest that even though the *cdc1-M78* mutation has the same effect as *cdc1-18* the former is not involved in the binding to Pol3. However, as the limits of the Pol3 binding region have not been delimited it is possible that this region is bigger or even that the binding to Pol3 requires more that one region. To further point to this last hypothesis it is interesting to note that mutant J7 cannot bind to Pol3 and is located at amino acid 393. However, there are other Cdc1 mutants that cannot bind to Pol3 and they are scattered along the length of Cdc1 (e.g. J10).

As mentioned above it is thought that the Pol3 binding region is involved in the binding to the A subunit. At present, the only evidence for this is that mutations in this region severely diminish or completely abolish binding. More concrete evidence is needed before it can be firmly established that the Pol3 binding region of the B subunit is indeed involved in the direct physical interaction with Pol3. An *in vitro* binding assay in which the binding of GST-tagged Pol3 to radioactively labelled Pol3 binding region is investigated would shed light on this. Further, using a series of slightly differently sized Pol3 binding region fragments of the B subunit it will help to narrow down the precise regions involved in the binding to Pol3. Until then, the region defined above as the Pol3 binding region shall remain as a region that is most likely but not definitely involved in the binding to the catalytic subunit.

5.4 Binding between the B and C subunits

There has been some contradictory data regarding the binding of the B subunit to the C subunit. Firstly, the data from MacNeill *et al.*, (1996) has suggested that the region of Cdc1 involved in the binding to Cdc27 is comprised of amino acids 25-50 at the N- terminus and amino acids 433-453 close to the C- terminus. However, the protein levels of the proteins were not investigated so it is possible that the observed differences in binding were due to the presence of different protein levels. A two-hybrid screen by E. Murray-Smith (a summer student in this lab) performed with Cdc27 as bait identified a clone of Cdc1 which was comprised of amino acids 157-462 only. In this thesis it was thought that the region of Cdc1 involved in the binding

to Cdc27 was DomIII (amino acids 120-158), as the Cdc1 mutants that fell in that region were unable to bind to Cdc27 in the two-hybrid system. This not only contradicted results by MacNeill *et al.*, (1996) but also those of E. Murray-Smith. The involvement of DomIII and some flanking region (from amino acids 108-171) in the direct binding to Cdc27 was however, disproved by further two-hybrid and Western analysis. Further, Western analysis of the mutants that fall within DomIII has shown that these mutants in the pBTM116 plasmid, in *S. cerevisiae* appear not to have detectable levels of protein. The exception is one mutant (J1) that appears to be present at a level that is detectable in a Western. Thus the region of Cdc1 involved in the binding to Cdc27 remains unclear. Given the results obtained by E. Murray-Smith it is possible that the region involved in the binding to Cdc27 is present in the C- terminal half of Cdc1. However, most of the mutations in the C- terminal region of Cdc1 investigated in this thesis appear not to affect the binding of Cdc1 to Cdc27. Therefore, it is possible that the Cdc27 binding region is present in the C- terminal half of Cdc1, and none of the mutations done in this study fell within the Cdc27 binding region. It is possible that the Cdc27 binding region is present in amino acids 433-453, as suggested by MacNeill *et al.*, (1996). This would fit with the data from E. Murray-Smith, and, as the most C- terminal mutation created in this study is only located at amino acid 393 (J7) it does not contradict data in this thesis. This region of Cdc1 has some homology with other B subunits as it includes part of conserved box X (see sequence alignment in Figure 1.3).

Overexpression of *cdc27*$^{+}$ was investigated (MacNeill *et al.,* 1996) and it was found that it does not rescue *cdc1-P13*. The *cdc1-P13* mutation is located at amino acid 389, and this suggests that probably the region around amino acid 389 in Cdc1 is not involved in the binding to Cdc27. The region around amino acid 389 is in conserved region VIII and is between mutants A8 and J7. The hypothesis that this region is not involved in the binding fits with the above hypothesis of the 433-453 region of Cdc1 being involved in the binding to Cdc27.

On the other hand the region of Cdc27 involved in the binding to Cdc1 has been identified (Reynolds *et al.*, 2000). It was found that the N- terminal 160 amino acids of Cdc27 is where the Cdc1 binding region is located.

5.5 Regions involved in Cdc1 function

The investigation, in chapter 4, into the ability of a range of Cdc1 mutants to rescue a *cdc1Δ* has identified a region of Cdc1 (DomIII) which is required for the rescue of the *cdc1Δ* strain. The mutants that fall within this region are not able to rescue when expressed to a level close to wild type. Of the three random mutants that are unable to rescue (with the promoter derepressed or repressed) two of them (J1 and J16) fall within or very close to this DomIII region. The protein levels of J1 and J16 were investigated, and it was found that they do have detectable levels of protein. The other mutants in this region were able to rescue when overexpressed so their protein level was not investigated. The mutants that fall within this DomIII region were investigated for the binding to Cdc27 and it was found that they were unable to bind to it. However, when the DomIII region on its own was investigated for the binding to Cdc27 it was found that it was not involved in the direct binding. The protein levels of the mutants were investigated again, as they were in a different plasmid to that used in the *cdc1Δ* rescue experiments and the binding assays are performed in *S. cerevisiae* and not in *S. pombe*. It was found that of all the mutants in this region only one of them (J1) has a protein level that can be detected by a Western blot. Hence, it appears that the inability of the mutants in DomIII to bind to Cdc27 might be due to the low level of protein present, but the inability of the mutants to rescue *cdc1Δ* is probably not due to protein levels.

Hence, the question arises as to why are the mutants within this region unable to rescue *cdc1Δ*. A plausible explanation is that this region is involved in the function of Cdc1. However, even though Cdc1 is essential its function is not known. Therefore, it is not possible to investigate this putative loss of function further.

Site directed mutant A3 is a single amino acid change that causes the Cdc1 protein to be unable to rescue a *cdc1Δ* and a *cdc1* t.s. Hence, it appears that this region could be important for the function of Cdc1. However, as this mutant is located in the region thought to be involved in the binding to Pol3 (see section 5.3) the phenotype seen with it is probably due to a lack of Pol3 binding.

5.6 Concluding Remarks

The main conclusions from this thesis can be summarised below:

- The second C- terminal zinc finger region of Pol3 (ZnF2) of both *S. pombe* and *S. cerevisiae* is involved in the direct interaction with the B subunit. The four cysteines of ZnF2 are involved in co-ordinating the zinc ion to maintain the structure of the zinc finger.

- The conserved region of Cdc1, DomIII, is not involved in the direct binding to Cdc27, but it is probably involved in the function of Cdc1.

- The region of Cdc1, from amino acid 293 to 329 (which may extended slightly into the C- terminus, see section 5.3) is thought to be involved in the binding to Pol3.

6 Materials and Methods

6.1 Materials

6.1.1 General Reagents

6.1.1.1 Antibiotics

Antibiotics were purchased from Sigma and used at the following concentrations:

Ampicillin	100 μg/ml
Chloramphenicol	15 μg/ml (liquid media)
	30 μg/ml (solid media)
Kanamycin	10 μg/ml
Tetracycline	5 μg/ml
Streptomycin	10 μg/ml

All stocks were made 1000x in dH_2O except for tetracycline which was made in ethanol. Also, chloramphenicol was made in ethanol at 34 mg/ml.

6.1.1.2 Chemicals

Unless stated otherwise chemicals were obtained from Sigma, Fisher or BDH laboratory supplies.

6.1.1.3 Enzymes

Enzymes were purchased from Promega, New England Biolabs, Roche or Sigma.

6.1.2 Buffers

Table 6.1 Common Buffers	
Buffer	Ingredients
TSB	10% PEG 3000 10 mM MgCl 10 mM $MgSO_4$ 5% DMSO in LB
TSBG	TSB + 20 mM glucose
Transfer Buffer	14.4 g glycine 3.0 g Tris 200 ml Methanol Up to 1l
PBS	11.5 g Na_2HPO_4 2.96 g NaH_2PO_4 5.84 g NaCl Up to 1l Autoclave
PBST	PBS + 0.1% Tween
50 x TAE	242 g Tris base 57.1 ml Glacial Acetic Acid 100 ml 500 mM EDTA, pH 8.0 Up to 1l
Protein Gel Buffer	30 g Tris 144 g Glycine 10 g SDS Up to 10 l
TE	10 mM Tris·HCl, pH8.0 1 mM EDTA, pH8.0 Final pH modified to 8.0
DNA Loading Buffer (6x)	0.25% Bromophenol Blue 0.25% Xylene cyanol FF 40% Sucrose
Sample Buffer (2x)	100 mM Tris-HCl, pH 6.8 200 mM DTT (added fresh prior to use) 4% SDS 0.2% Bromophenol blue

6.1.3 Oligonucleotides

Oligonucleotides were obtained from Amersham Pharmacia Biotech and Sigma Genosys.

Table 6.2 Oligonucleotides for amplification and sequencing		
Oligo Name	Description	5' to 3' Sequence
UPBR322@370	For pBR322 sequencing	TCATGGCGACCACACCCGTC
LPBR322@390	For pBR322 sequencing	TGATGCCGGCCACGATGCGTC
CDC15'U	Cloning Ccd1 5'	CATTTACTTGCAATACTCGCTCG T
CDC15'L	Cloning Ccd1 5'	CGACCTTGTTCATCTTCATGGCC C
CDC13'U	Cloning Ccd1 3'	ATGCTCTTGAGGATGACTATGGC C
CDC13'L	Cloning Ccd1 3'	GCTCACAGCCTACGCATTAGCG GA
SEQ1U	Sequencing Cdc1 located at nt. 953, upper strand	AAATCAAAAGTTTGGTTGGG
SEQ2L	Sequencing Cdc1 located at nt. 1398, lower strand	GAAGTAATTGGAGTCTCTGC
POL3CT1	Cloning of Pol3 C-terminus	GATACAGCTCGGATCCTTGGTG AAAAAGCTAGCTCTTTAC
PACT5	Amplification of insert in pACT2	CGCGTTTGGAATCACTACAGGG ATG
PACT3	Amplification of insert in pACT2	GAAATTGAGATGGTGCACGATG CAC
SEQ2HYB	2 hybrid sequencing primer, in pACT	GGCTTACCCATACGATGTTC
nmt5'	Primer in the 5' of the nmt promoter	GGAATCCTGGCATATCATCATCA ATT
nmt3'	Primer in the 3' of the nmt terminator	GCAGCTTGAATGGGCTTCCATA GT
pGEX5'	Primer to the 5' of pGEX vector MCS	GCAAGCCACGTTTGGTG
pGEX3'	Primer to the 3' of pGEX vector MCS	CCGGGAGCTGCATGTGTCAGAG G
BTM3'	Primer to the 3' of pBMT116	ACCTGAGAAAGCAACCTGACC
pBTM116 5'	Primer to the 5' of pBMT116	CTTCGTCAGCAGAGCTTC
Pol31SEQ1	Sequencing of Pol31	CGTACTGGACGAAGTGATC
Pol31SEQ2	Sequencing of Pol31	CCACCTATTTCCACTACTC
ScPol3 5'	Cloning *S. cerevisiae* Pol3	CGCGCCCGGGTATACATATACG CACAGCA
ScPol3 3'	Cloning *S. cerevisiae* Pol3	GGCCGAATTCTGATGTGCCACCC TATCG
ScPol3 A	Cloning *S. cerevisiae* Pol3	CTAAGATCCTACACG
ScPol3 B	Cloning *S. cerevisiae* Pol3	CTAAAAAGTGCGCAG
ScPol3ΔZnF2	Cloning of *S. cerevisiae*	GGCCGAATTCATCTCTGACATCG

	Pol3, with ZnF2 deleted	TATAATGCC
ScPol3SEQ1	Sequencing of *S. cerevisiae* Pol3	CATTCGAGCGTAAG
ScPol3SEQ2	Sequencing of *S. cerevisiae* Pol3	GTAAACACATTACG
ScPol3SEQ3	Sequencing of *S. cerevisiae* Pol3	TTATGATGGCGCAC
ScPol3SEQ4	Sequencing of *S. cerevisiae* Pol3	CGCCTGTTTGTCGC
DomIII F	Amplification of DomIII region in Cdc1, Forward	GGCCGGATCCAAGAAGATGCTG CTTCAGG
DomIII B	Amplification of DomIII region in Cdc1, Backward	GGCCGGATCCAATGGAATGAGA GAAG
Cdm1MOD5	Amplification of Cdm1	GGGGATCCTATGAAGAAGCGCA CTACTC
Cdm1B2	Sequencing Cdm1	CACCCTGGTAGGAGGGGACC
ZnF2F	Primer in the 5' end of ZnF2	CTCAAGATTATGGAC

Table 6.3Oligonucleotides for mutagenesis and plasmid modification		
Oligo Name	Description	5' to 3' Sequence
AANO1	Used for site directed mutagenesis of Cdc1	TAGATGTCACTTTAATGGCTGGT CCTTATG
AANO2	Used for site directed mutagenesis of Cdc1	GTCACTTTAATGCCTGCTCCTTA TGATTAC
AANO3	Used for site directed mutagenesis of Cdc1	CCTGGTCCTTATGCTTACAGTTC AACTATC
AANO4	Used for site directed mutagenesis of Cdc1	GTTCAACTATCCTTGCTCAACAG CCTTTGC
AANO5	Used for site directed mutagenesis of Cdc1	ACAAACAGTTACGGCTCCCACTT GGCTTTC
AANO6	Used for site directed mutagenesis of Cdc1	AAACAGTTACGAATGCCACTTG GCTTTCTC
AANO7	Used for site directed mutagenesis of Cdc1	TGGCTACTAGCGCCCAAAACAT TAATGATC
AANO8	Used for site directed mutagenesis of Cdc1	AATCATATCACAGCTACCAGCC CTGATACC
AANO1/2	Used for site directed mutagenesis of Cdc1	GTCACTTTAATGGCTGCTCCTTA TGATTAC
AANO5/6	Used for site directed mutagenesis of Cdc1	ACAAACAGTTACGGCTGCCACT TGGCTTTC
ARG1F	Site directed mutagenesis of ZnF2 of Pol3/Cdc2	GAGGAAAAATACTCAGCATTAT GGACACAATG
ARG1B	Site directed mutagenesis of ZnF2 of Pol3/Cdc2	CATTGTGTCCATAAATGCTGAGT ATTTTTCCTC
TRPF	Site directed mutagenesis	AATACTCAAGATTAGCGACACA

	of ZnF2 of Pol3/Cdc2	ATGCCAAAG
TRPB	Site directed mutagenesis of ZnF2 of Pol3/Cdc2	CTTTGGCATTGTGTCGCTAATCT TGAGTATT
GLUF	Site directed mutagenesis of ZnF2 of Pol3/Cdc2	GTAACTTACATAGTGCAGTTTTG TGTTCAA
GLUB	Site directed mutagenesis of ZnF2 of Pol3/Cdc2	TTGAACACAAAACTGCACTATG TAAGTTAC
PHETYRF	Site directed mutagenesis of ZnF2 of Pol3/Cdc2	AGAACTGTGACATTGCTGCTATG CGGGTTAAGG
PHETYRB	Site directed mutagenesis of ZnF2 of Pol3/Cdc2	CCTTAACCCGCATAGCAGCAAT GTCACAGTTCT
ARG2F	Site directed mutagenesis of ZnF2 of Pol3/Cdc2	GACATTTTTTATATGGCGGTTAA GGTTAAAA
ARG2B	Site directed mutagenesis of ZnF2 of Pol3/Cdc2	TTTTAACCTTAACCGCCATATAA AAAATGTC
CYS1F	Site directed mutagenesis of ZnF2 of Pol3/Cdc2	GATTATGGACACAAKCCCAAAG GTGCGCTGG
CYS1B	Site directed mutagenesis of ZnF2 of Pol3/Cdc2	CCAGCGCACCTTTGGGMTTGTG TCCATAATC
CYS2F	Site directed mutagenesis of ZnF2 of Pol3/Cdc2	CACAATGCCAAAGGKCCGCTGG TAACTTAC
CYS2B	Site directed mutagenesis of ZnF2 of Pol3/Cdc2	GTAAGTTACCAGCGGMCCTTTG GCATTGTG
HISF	Site directed mutagenesis of ZnF2 of Pol3/Cdc2	GCGCTGGTAACTTAKCTAGTGA AGTTTTGTG
HISB	Site directed mutagenesis of ZnF2 of Pol3/Cdc2	CACAAAACTTCACTAGMTAAGT TACCAGCGC
CYS3F	Site directed mutagenesis of ZnF2 of Pol3/Cdc2	CATAGTGAAGTTTTGKCTTCAAA TAAGAACTG
CYS3B	Site directed mutagenesis of ZnF2 of Pol3/Cdc2	CAGTTCTTATTTGAAGMCAAAA CTTCACTATG
CYS4F	Site directed mutagenesis of ZnF2 of Pol3/Cdc2	GTTCAAATAAGAACKCTGACAT TTTTTATATGC
CYS4B	Site directed mutagenesis of ZnF2 of Pol3/Cdc2	GCATATAAAAAATGTCAGMGTT CTTATTTGAAC
SpCys1AF	Site directed mutagenesis of ZnF2 of *S. pombe* Pol3	CGCTTATGGACTCAAGCTCAGC GATGCCAAGG
SpCys1AB	Site directed mutagenesis of ZnF2 of *S. pombe* Pol3	CCTTGGCATCGCTGAGCTTGAGT CCATAAGCG
SpCys2AF	Site directed mutagenesis of ZnF2 of *S. pombe* Pol3	CTCAATGTCAGCGAGCCCAAGG AAGTATGC
SpCys2AB	Site directed mutagenesis of ZnF2 of *S. pombe* Pol3	GCATACTTCCTTGGGCTCGCTGA CATTGAG
SpCys3AF	Site directed mutagenesis of ZnF2 of *S. pombe* Pol3	CATCAAGACGTCATTGCTACCA GTAGAGACTG
SpCys3AB	Site directed mutagenesis of ZnF2 of *S. pombe* Pol3	CAGTCTCTACTGGTAGCAATGAC GTCTTGATG

SpCys4AF	Site directed mutagenesis of ZnF2 of *S. pombe* Pol3	GTACCAGTAGAGACGCCCCCAT ATTTTATATG
SpCys4AB	Site directed mutagenesis of ZnF2 of *S. pombe* Pol3	CATATAAAATATGGGGGCGTCT CTACTGGTAC
SpCys4SF	Site directed mutagenesis of ZnF2 of *S. pombe* Pol3	GTACCAGTAGAGACTCCCCCAT ATTTTATATG
SpCys4SB	Site directed mutagenesis of ZnF2 of *S. pombe* Pol3	CATATAAAATATGGGGGAGTCT CTACTGGTAC
Sdp5F	For mutation of Pol31 into Sdp5	CTAGCCGTTTCAGGCGAAAATA TCAATGAT
Sdp5B	For mutation of Pol31 into Sdp5	ATCATTGATATTTTCGCCTGAAA CGGCTAG
pBTMMOD1	For modification of the polylinker of pBTM116	3GATCCGATCCGGCGGCCGCTTG CA
pBTMMOD2	For modification of the polylinker of pBTM116	3AGCGGCCGCCGGATCG

6.1.4 Plasmids

Table 6.4 Plasmids used in this thesis

Plasmid Name	Description	Source
pBTM116	Two-hybrid vector. Sequences inserted will be produced as a LexABD protein fusion.	(MacNeill *et al.*, 1996)
pBTM-Cdc1	Cdc1 in two-hybrid vector. Produces LexABD-Cdc1.	(MacNeill *et al.*, 1996)
pBTM-Cdc1Δ453	Cdc1 with the last 10 amino acids deleted in a two-hybrid vector produces LexABD-Cdc1Δ453	(MacNeill *et al.*, 1996)
pBTM116-Pol31	Pol31 in a two-hybrid vector. Produces LexABD-Pol31	(MacNeill *et al.*, 1996)
pACT2	Two-hybrid vector. Sequences inserted will be produced as Gal4AD protein fusions.	(Reynolds *et al.*, 2000)
pACT2-Pol3	Full length *S. cerevisiae* Pol3 in pACT2. Produces Gal4AD-Pol3.	This study.
pACT2-ΔZnF2	*S. cerevisiae* Pol3 with the second C- terminal zinc finger deleted. Produces Gal4AD-ΔZnF2.	This study.
pACT2-Pol3CT	C- terminus of *S. pombe* Pol3 in two-hybrid vector. Produces Gal4AD-Pol3CT.	This study and this lab.
pACT-ZnF2	*S. pombe* ZnF2 in a two-hybrid vector. Produces Gal4AD-ZnF2	This lab and this study.
pGAD	Two-hybrid vector. Sequences inserted will be produced as	(MacNeill *et al.*, 1996)

	Gal4AD protein fusions.	
pGAD-Pol3	Full length *S. pombe* Pol3 in pGAD. Produces Gal4AD-Pol3.	This lab.
pGAD-Cdc27	Cdc27 in a two-hybrid vector. Produces Gal4AD-Cdc27.	(MacNeill *et al.*, 1996)
pAA	Vector used in "three hybrid" assays. Sequences inserted will be produced as myc-tagged protein fusions.	(Reynolds *et al.*, 2000)
pAA-Cdc27	Vector used in "three hybrid" assay. Produces myc-tagged Cdc27.	(Reynolds *et al.*, 2000)
pAA-Cdm1	Cdm1 for use in three hybrid assay. Produces myc-tagged Cdm1.	This study.
pBR322-Cdc1	Plasmid used for the pentapeptide insertion mutagenesis system.	This lab.
pREP3xH_6BN	pREP vector. Sequences inserted in the multiple cloning site will be under the control of the nmt1 promoter. Protein produced will have both a H6 and an MRGS tag.	This lab.
pREP3xH_6BNA1-A10	Site directed Cdc1 mutants in pREP3xH_6BN.	This study.
pREP3xH_6BNJ1-J22 (there is no J14)	Pentapeptide insertion mutagenesis Cdc1 mutants in pREP3xH_6BN.	This study.
pTZ19R	General cloning vector.	Pharmacia
pREP81X-Pol3	Full length *S. pombe* Pol3 in pREP81X plasmid.	This lab.
pGEX6P1B-ZnF2	GST tagged *S. pombe* ZnF2.	This lab.

6.1.5 Antibodies

Gal4 AD monoclonal antibody was purchased from BD Biosciences (Clontech); LexA antibody was purchased from Santa Cruz Biotechnologies; Anti-RGS•His Antibody was purchased from Qiagen. Anti-Cdc1 antibody was described in Zuo *et al.*, (1997).

6.1.6 Bacterial and Yeast Media

All media recipes below are for liquid media. For solid media 10 g of agar was added per 500ml before autoclaving.

6.1.6.1 Bacteria Media

Table 6.5 Bacteria Media	
Media	Ingredients
LB	1% (w/v) Bacto-tryptone 0.5% (w/v) Yeast extract 0.5% (w/v) NaCl pH was adjusted to 7.2 with NaOH
10x M9 salts	6% (w/v) Na_2HPO_4 3% (w/v) KH_2PO_4 0.5% (w/v) NaCl 1% (w/v) NH_4Cl
M9-Leu	10% (w/v) -LEU dropout mix 0.2% (w/v) Glucose 10% (v/v) 10x M9 salts 0.024% (w/v) $MgSO_4$ 0.022% (w/v) $CaCl_2$
SOC	2% (w/v) Bacto-tryptone 0.5% (w/v) Yeast extract 0.06% (w/v) NaCl 0.02% (w/v) KCl 0.1% (w/v) $MgCl_2$ 0.12% (w/v) $MgSO_4$ 0.4% (w/v) Glucose

6.1.6.2 Yeast Media

For *Schizosaccharomyces pombe* Yeast Extract (YE) was used for vegetative growth, Edinburgh Minimal Media (EMM) for minimal media and Malt Extract (ME) as sporulation media (reviewed in Moreno *et al.,* 1991). To check if *S. pombe* was diploid or haploid, phloxin B was added to YE solid media to a final concentration of 2.5 mg/litre.

For *Saccharomyces cerevisiae* media YMM was used for minimal media and YPDA for vegetative growth. YMM is 0.67% (w/v) Yeast Nitrogen Base (Difco), 2% (w/v) Glucose and 1x CSM (Bio101). CSM is complete supplements minus the desired selection and was made in a 20x stock, filter sterilised and the pH was adjusted to 7.0. It was used as 10x. YPDA is 1% (w/v) Yeast extract, 2% (w/v) Bacto-peptone, 2% (w/v) Glucose, 0.003% (w/v) Adenine sulphate.

6.1.7 Bacterial Strains

Table 6.6 Bacterial Strains used in this thesis		
Strain	Genotype	Source
JM109	e14⁻(McrA-) reaA1 endA1 gyrA96 thi-1hsdR17(r_K-m_K+) supE44 relA1 Δ(lac-proAB) [F'traD36 proAB $lacI^qZ\Delta M165$]	
DH5α	F-, ø80dlacZDM15, D(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rk-, mk+), phoA, supE44, l-, thi-1, gyrA96, relA1	
MC1066	pyrF74::Tb5(km^r) leuB6 ara^+ trpC9830 Δ(ara leu), Δ(lacIPOZYA)X74 galU galK $StrA^r$	
FH1046	Strain containing plasmid pHT385, which contains Tn4430Ω5, for use in pentapeptide insertion mutagenesis.	(Hallet *et al.*, 1997)
DS941	Recipient strain for pentapeptide insertion mutagenesis	(Hallet *et al.*, 1997)

JM109 and DH5α were routinely used for transformations (see section 6.2.1.1), whereas MC1066 was used for transforming by electroporation (see section 6.2.1.2 and 6.2.1.3).

6.1.8 Yeast Strains

Table 6.7 *S. pombe* strains used in this thesis		
Strain	Genotype	Source
cdc1-223	*cdc1-223, his2, leu1,* h^+	Hiroshi Kondoh, Kyoto
cdc1-A24	*cdc1-A24, leu-32, ura-D18*	(Reynolds *et al.*, 2000)
cdc1-64	*mis1-64, ade6-704, leu1-32, h-*	Hiroshi Kondoh, Kyoto
cdc1-P13	*cdc1-P13 leu1-32* h^+ *(Sp81)*	(Nurse *et al.*, 1976)
cdc1Δ	*cdc1::ura4*$^+$*/cdc1*$^+$ *leu-32/leu1-32 ura4-D18/ura4-D18 ade6-M210/M216 h-/h+*	(MacNeill *et al.*, 1996)
ED1090	*leu1-32, ura4-D18,* h^-	This lab.

Table 6.8 *S. cerevisiae* strains used in this thesis		
Strain	Genotype	Source
L40	*MAT a, ade2, trp1-901, leu2-3,-112, his3Δ200, lys2-801am, URA3::(lexAop)$_8$-LacZ, LYS2::(lexAop)$_4$-HIS3*	(Hollenberg *et al.*, 1995)
Y187	*MAT α, gal4-542, gal80-538, ade2-101, his3Δ200, trp1-901, leu2-3-112, ura3-52, URA3::GAL1-lacZ*	Clontech
CTY10-5d	*MATa, ade2, met$^-$, trp1-901, leu2-3,112, his3-200, gal4-gal80-URA3::lexA-lacZ*	(MacNeill *et al.*, 1996)

6.2 Methods

6.2.1 General Methods

6.2.1.1 Transformation of *E. coli*

In this method *E. coli* were grown, made competent and transformed immediately.

1) TSB (see Table 6.1) was freshly made and chilled on ice.
2) *E. coli* cells were grown in 50 ml of LB at 37°C with shaking to an OD_{600} of 0.4-0.5.
3) Cells were centrifuged in a bench top centrifuge at 3000 rpm for 10 min.
4) Cells were resuspend in 1/10 volume of TSB and chilled on ice for a minimum of 10 min.
5) For transformation 100 µl of cells were added to ≥100 pg of DNA.
6) Cells were incubated on ice for 30min.
7) 200 µl of TSBG was added and incubated with shaking at 37°C for 1 hour.
8) 300 µl was spread on an appropriate selective plate.

6.2.1.2 Preparation of electrocompetent E. coli

In this method *E. coli* were made electrocompetent and stored at –80°C for transforming at a later time.

1) A 5 ml miniculture of the desired *E. coli* strain was grown in LB overnight at 37°C. This miniculture was used to inoculate 500 ml of LB liquid pre-warmed to 37°C to an OD_{600} of 0.1.
2) Cells were grown at 37°C with shaking until OD_{600} reached 0.6.
3) Cells were chilled on ice for 15 min then centrifuged in prechilled bottles at 4200 rpm for 20 min at 4°C.
4) Cells were washed twice in chilled sterile dH_20.
5) Step 4 was repeated with chilled 10% (v/v) glycerol.
6) Cells were resuspend in 2 ml of chilled 10% glycerol, and aliquoted into Eppendorf tubes on ice (40 µl aliquots).
7) Cells were frozen in liquid nitrogen and stored at -80°C.

6.2.1.3 Transformation of electrocompetent *E. coli*

1) An aliquot of the electrocompetent cells (see section 6.2.1.2) was thawed on ice.
2) 100 ng of DNA was added to the cells.
3) Cells were transferred to a prechilled electroporation cuvette, with a 0.2 cm gap.
4) Cells were pulsed at 200 Ohms, 25 µF and 2.5 kV, using a Bio-Rad Gene Pulser II, and making sure that the cuvette base is dry.
5) Immediately after electroporating, 1 ml of SOC medium (see section 6.1.6.1) was added.
6) Cells were transferred to an Eppendorf tube and incubated with shaking for 1 hour at 37°C.
7) Cells were centrifuged in a microfuge at 14,000 rpm for 30 seconds.
8) Supernatant was discarded and the pellet was resuspended in 100 µl of SOC medium (see Table 6.5).
9) Cells were spread on selective plates.

6.2.1.4 Mating *E. coli*

E. coli strains were mated routinely by this method. The strains to be mated were scraped from a plate using a toothpick, were mixed into a spot of dH_2O on an LB

plate and the spots were airdried. The plate was then incubated at 37°C for three hours. The mated cells were typically then scraped, resuspended into LB liquid and plated.

6.2.1.5 Extraction of Plasmids from *E. coli*

For routine plasmid extraction from *E. coli* the Qiagen miniprep (typically 5-15 μg of DNA were obtained with this method) kit was used, which gave DNA of suitable quality for sequencing or any other use. For larger DNA preps either a midiprep (typically up to 100 μg of DNA were obtained with this method) or a maxiprep kit (typically up to 500 μg of DNA were obtained with this method) from Qiagen were used. The manufacturers instructions within the kits were followed.

6.2.1.6 Transformation of *S. pombe*

This method for high efficiency transformation of *S. pombe* is based on that described by Prentice (1992). Care must be taken to ensure that the electrocompetent cells are in contact with the DNA for as little time as possible as this may result in degradation of the DNA. Also, ice cold sorbitol must be added to the cuvette as quickly as possible after the electroporation, delay in doing this will result in reduced transformation efficiencies.

1) Strain to be transformed was grown in 200 ml of selective media to an OD_{600} of 0.5 at 32°C with shaking.
2) Cells were centrifuged at 3000 rpm in a benchtop centrifuge for 4 minutes.
3) Cells were washed three times with 50 ml of ice cold 1.2 M sorbitol.
4) Cells were resuspended in 1/100 of the culture volume of ice cold 1.2 M sorbitol.
5) Just prior to electroporation, 200 μl of cells were mixed with 1 ng to 1 μg of DNA and transferred to an ice cold electroporation cuvette with a 0.2 cm gap.
6) Cells were pulsed at 2.25 kV, 200 Ω, and 25 μF.
7) Immediately after pulsing 500 μl of ice cold 1.2 M sorbitol was added to the cells.

8) Cells were plated on selective media.

6.2.1.7 Mating *S. pombe*

A drop of dH_20 was placed on an ME plate. Both strains to be mated were scraped from a plate with an inoculation loop and were inoculated and mixed on the drop of dH_20. As a control the individual strains were patched at either side of the drop of dH_20. This was incubated at the appropriate temperature for two days. After the two days the patch was checked for the existence of asci which are indicative of mating. The control patches were also checked for the absence of asci, and hence absence of homothallic *S. pombe*.

6.2.1.8 Sporulation and helicase treatment

For sporulation of *S. pombe*, diploid cells were grown on ME (see section 6.1.6.2) and spores were typically seen after 2-4 days. After sporulation cells were routinely treated with helicase to kill any vegetative cells. For helicase treatment 1 ml of dH_20 was inoculated with a loopful of culture. To this 20 µl of a 1in10 dilution of helicase (*Helix pomatia* juice) was added and incubated overnight at 25-29°C. The spores were then washed three times with dH_20 and plated on appropriate media.

6.2.1.9 Transformation of *S. cerevisiae*

This method was routinely used to transform plasmids into *S. cerevisiae*, usually as a first stage to two-hybrid screening. In the cases where more than one plasmid was needed in the same strain for the two-hybrid assay the plasmids were routinely co-transformed with this method.

1) With a loop, several colonies were inoculated into 1 ml of YPD.
2) Cells were vortexed to resuspend them.
3) The cell suspension was added to a 250 ml flask containing 50 ml of YPD.
4) Cells were incubated at 30°C overnight, with shaking, until stationary phase.

5) The 50 ml overnight culture was transferred to a 500 ml flask containing 300 ml of YPD. This was incubated for 3 hr at 30°C with shaking.
6) Cells were centrifuged at 3000 rpm in a benchtop centrifuge for 5 min at room temperature. Supernatant was discarded.
7) Pellet was resuspended in 50 ml dH_20.
8) Step 6 was repeated.
9) Pellet was resuspended in 1 ml fresh 1x TE/LiAc.

- 100 µl 10xTE
- 100 µl 1M Lithium Acetate (LiAc)
- 800 µl H_20

10) PEG/LiAc solution was prepared.

- 8 ml 50% PEG
- 1 ml 10x TE
- 1 ml 1M LiAc

11) The following was added to a 1.5 ml Eppendorf tube: 0.1 µg DNA, 0.1 mg Herring testes DNA (Clontech). This was mixed with a pipette.
12) 100 µl of yeast competent cells (from step 9) was added and mixed.
13) 600 µl PEG/LiAc was added and mixed by vortexing.
14) This was incubated with shaking for 30 min at 30°C.
15) 70 µl of DMSO was added.
16) Cells were heat shocked at 42°C for 15 min, then chilled on ice for 10 min.
17) Cells were pelleted in a microfuge at top speed for 2 sec and the supernatant was removed.
18) Cells were resuspended in 500 µl 1x TE
19) Cells were plated on selective media.

6.2.1.10 Genomic DNA or plasmid preparation from *S. cerevisiae*

Essentially the same protocol was used to rescue plasmid DNA from *S. cerevisiae* and to extract genomic DNA, the only difference being the final step.

1) 10 ml of cells were grown in the appropriate media at 30°C with shaking overnight so they reached midlog phase.
2) Cells were centrifuged at 3000 rpm in a benchtop centrifuge for 3 min and washed twice with 10 ml of dH_20.
3) Cell were transferred into a 1.5 ml Eppendorf tube and centrifuged at top speed in a microfuge for 15 s and the supernatant was discarded.
4) To the tube 300 mg of glass beads, 200 µl lysis solution and 200 µl of phenol:chloroform:isoamyl alcohol (ratio 25:24:1) was added.

Lysis solution:

- 2% (v/v) Triton X-100
- 1% (w/v) SDS
- 100 mM NaCl
- 10 mM Tris-HCl, pH8.0
- 1 mM EDTA

5) The tube was vortexed for 5 min.
6) 200 µl of dH_20 was added and the tube was vortexed briefly.
7) The tube was centrifuged at top speed in a microfuge for 3 min, the upper phase was transferred to a fresh tube.
8) The DNA was precipitated by doing an ethanol precipitation (see section 6.2.1.13) and the pellet was air dried.
9) In the case of plasmid DNA rescue, the pellet was resuspended in 20 µl of dH_20 and 0.5 µl was used to transform electrocompetent *E. coli* (see section 6.2.1.3).
10) For genomic DNA extraction the pellet was resuspended in 100 µl of TE and 2 µl was used as template for PCR (see section 6.2.1.15) in a total volume of 50 µl.

6.2.1.11 DNA Electrophoresis

Agarose gel electrophoresis was typically performed using 50 ml of 0.8-1.5% multipurpose (MP) Agarose gels in 1x TAE buffer (see Table 6.1) with 10 µg ethidium bromide. DNA loading buffer (see Table 6.1) was added to the samples to 1x. Electrophoresis was typically performed for 1 hour at 120V in approximately 800 ml 1xTAE with 10 µg ethidium bromide, the DNA was visualised on a Hewlab UVT-28M UV light box.

6.2.1.12 Phenol Chloroform Extraction

This method was used to clean up DNA. An equal amount of phenol:chloroform:isoamyl alcohol (ratio 25:24:1) was added to a solution of DNA. This was then vortexed and centrifuged at top speed in a microfuge for 1 min. The top aqueous layer containing the DNA was removed to a fresh tube and routinely an ethanol precipitation was done (see section 6.2.1.13).

6.2.1.13 Ethanol Precipitation of DNA

This method was usually used to clean up DNA and precipitate it out of solution to resuspend in the desired buffer. 2.5 volumes of 95% ethanol and 1/10 volume sodium acetate pH 5.2 was added to the DNA solution. This was vortexed and incubated in ice for 10 minutes. The tube was then centrifuged at top speed in a microfuge for 20 minutes. The pellet was washed with 500 µl of 70% ethanol, dried in a speed vac and resuspended in a suitable volume of an appropriate buffer. TE (see Table 6.1) was routinely used.

6.2.1.14 Purification/ Extraction of DNA fragments

This method was routinely used to extract and purify DNA from an agarose gel, typically after restriction digests (see section 6.2.1.17). Following gel electrophoresis DNA fragments were excised from agarose gels. The DNA was then extracted using

a Qiaquick gel extraction kit (Qiagen) following the manufacturers instructions. This method was also used for the purification of DNA after methods such as the removal of phosphate ends from linear DNA (see section 6.2.1.18).

6.2.1.15 Polymerase Chain Reaction

PCR amplification reactions were typically performed in a total volume of 20 - 60µl. A typical reaction mix is as follows: 1 µl primers (100 mM), 0.4 µl dNTPs (10 mM), 100 ng template, 1x appropriate buffer (supplied with the enzyme as 10x stock), 0.21 units enzyme (typically Deep Vent, *Pfu* or *Taq* polymerase) and water to 20 µl were added and mixed. Typically *Taq* was used for diagnostic PCR whereas a proof-reading enzyme, either Deep Vent or *Pfu* was used for amplification prior to cloning. The reaction was placed in a 0.2 ml tube in either a Techne Genius or a Techne Techgene cycler; unless otherwise stated the reaction conditions were:

- 94°C 1min 30s
- 55°C 1min 30s
- 72°C 3min
- 30 cycles
- 10°C Hold

6.2.1.16 Sequencing

Sequencing was performed with either dRhodamine or BigDye chain terminators (Perkin Elmer). In either case 100-250 ng of DNA was added to 1.6 pmol of primer and 4 µl of the Big Dye or dRhodamine mix. This was then placed in either a Techne Genius or a Techne Techgene cycler with the following programme:

- 96°C 30 seconds
- 50°C 15 seconds
- 60°C 4 minutes
- 25 cycles

- Hold at 10°C

The reaction was then cleaned up by ethanol precipitation (see 6.2.1.13) without resuspending the final pellet or by using a Genetix genCLEAN dye terminator removal column and following the manufacturers instructions. If a Genetix genCLEAN column was used the final eluate was evaporated by incubating at 100°C for 5-10 min or until completely evaporated. Sequencing analysis was performed by in the house sequencing service on an ABI PRISM DNA sequencer. The sequences were analysed using Gene Jockey II and Sequencher, both in a Macintosh.

6.2.1.17 Restriction Enzyme Digests

Restriction enzyme digests were done following manufacturers instructions. Typically they are performed in a total volume of 60µl in 1x buffer and with 1µl of enzyme (10-20 units) and 1µg of DNA.

6.2.1.18 Phosphate End Removal

The removal of phosphate groups at the 5' end of linear molecules was routinely performed prior to ligation in order to avoid the re-circularisation of the vector DNA. This was achieved by incubating 0.5 units of Shrimp Alkaline Phosphate (SAP) obtained from USB with the linearised DNA (typically 1 µg after restriction digest; see section 6.2.1.17) at 37°C in either 1x SAP buffer (supplied with the enzyme) or in 1x restriction enzyme buffer for 1 hr. Prior to use in ligation the end product was purified using a Qiaquick Gel extraction Kit column (Qiagen; see section 6.2.1.14).

6.2.1.19 Ligations

Ligations were typically carried out in a total volume of 20µl. Vector and insert DNA to be ligated were mixed according to the following formula:

$$\mu g \text{ of Insert} = \frac{\mu g \text{ of vector} \times \text{kb size of insert}}{} \times \text{molar ratio}$$

kb size of vector

A 1:3 ratio of vector:insert was typically used. 1 unit of ligase and ligase buffer to 1x were added. This reaction was incubated at 16°C overnight, although it can be left over 48 hours. Half of the ligation was then transformed into a suitable *E. coli* strain as described in 6.2.1.1.

6.2.1.20 Primer Annealing

This method was used for the annealing of two primers to each other. Annealed primers were usually used when the polylinker of a plasmid was to be modified.

1) Equal amounts of both primers were added to an Eppendorf tube.
2) The sample was boiled in a beaker of boiling dH_20 for 2 min.
3) The heat was removed and the boiling dH_20 was allowed to cool to room temperature. The dH_20 was left to cool slowly, so primers had time to anneal to each other.
4) Once the dH_20 is at room temperature the primers were annealed and ready to use.

6.2.1.21 Protein Electrophoresis

Electrophoresis of proteins was typically performed in 8-15% acrylamide gels. The apparatus used was obtained from Bio-Rad (mini protean II) and was assembled as in the manufacturers instructions. Below is a recipe for a typical gel:

Separating Gel (12%):

dH_20	2.10ml
1.0 M Tris-HCL, pH8.8	3.75ml
10% SDS	100µl
10% AMPS	50µl

Acrylamide/Bis (30% Stock)	4ml
Temed	5µl

Stacking Gel (5%):

dH_20	7.35ml
1.0 M Tris-HCL, pH6.8	1.25ml
10% SDS	100µl
10% AMPS	50µl
Acrylamide/Bis (30% Stock)	1.3ml
Temed	10µl

Typically sample buffer to 1x (see Table 6.1) was added to 10 µg of protein, they were loaded on the gel and typically run for 1 hr at 200 V in running buffer (see Table 6.1).

6.2.1.22 Total protein extraction from *S. cerevisiae*

This method was used to extract total protein from *S. cerevisiae* for Western analysis. It was used extensively to investigate the protein levels of two-hybrid fusion proteins in the host strains. The plasmid expressing the protein was transformed into the appropriate *S. cerevisiae* strain (see section 6.2.1.9) and the method below was followed.

1) A 5 ml culture of the desired strain was grown in selective media overnight at 30°C with shaking.
2) The OD_{600} of the culture was measured.
3) An equivalent of 3 OD_{600} units was centrifuged in a benchtop centrifuge at 3000 rpm for 3 min.
4) The supernatant was discarded and the pellet was resuspended in 500 µl of 0.2 M NaOH and incubated on ice for 10 min.
5) 50 µl of 50% TCA was added to the sample and was incubated on ice for another 10 min.

6) The sample was centrifuged at top speed in a microfuge for 3min.
7) The supernatant was discarded and the pellet was resuspended in 35 µl of dissociation buffer.

Dissociation buffer:

- 500 µl 1 M Tris, pH6.8
- 40 µl 500 mM EDTA pH8
- 2 ml 10% SDS
- 1 ml glycerol
- 100 µl β-mercaptoethanol
- 1.36 ml dH_20

8) 15 µl of 1 M unbuffered Tris was added to the sample.
9) Sample was heated at 100°C for 10 min and centrifuged for 1 min at top speed in a microfuge.

The supernatant is the total protein. Typically the total protein concentration was determined (using a Bio Rad protein assay kit, see section 6.2.1.23). 4 µl of Blue Dissociation Buffer (as dissociation buffer but with 10 mg bromophenol blue powder added) was added to an estimated 10 µg of total protein which was subjected to protein electrophoresis (see section 6.2.1.21), prior to Western analysis (see section 6.2.1.25).

6.2.1.23 Bio Rad Protein Assay Kit

The kit is used for the measurement of protein concentration and was routinely used to measure the protein concentration of total protein extracted from *S. cerevisiae*, before Western analysis. The samples were routinely in dissociation buffer (see section 6.2.1.22). The high SDS concentration will interfere with the assay, hence, it was diluted down 1/1600. 0.5 µl of protein from the total protein extraction from *S. cerevisiae* method (section 6.2.1.22) was diluted in 800 µl of H_2O. To this 200 µl of the undiluted Bio Rad dye reagent concentrate was added and was incubated at room

temperature for 5min. After the incubation the OD_{595} was measured and the measurement was compared to a standard curve using BSA as standard.

6.2.1.24 Total protein extraction from *S. pombe*

This method was used for the total protein extraction of *S. pombe* for use in Western analysis.

1) 50 ml cultures were grown to OD_{600} = 0.2 - 0.4 in EMM at 32°C.
2) Samples with equal OD_{600} were centrifuged at 3000 rpm in a benchtop centrifuge for 4 min.
3) Pellet was resuspended in 5 ml of ice-cold STOP buffer

STOP Buffer:

- 150 mM NaCl
- 50 mM NaF
- 10 mM EDTA
- 1 mM NaN_3

4) Samples were centrifuged at 3000 rpm in a benchtop centrifuge for 4 min.
5) Pellet was resuspended in 100 µl of Buffer A:

Buffer A:

- 10 mM sodium phosphate Buffer pH7.0
- 1x Complete Inhibitors (Roche)
- 1% Triton X-100
- 0.1% SDS
- 1 mM EDTA pH8.0
- 150 mM NaCl
- 1 mM PMSF (to be added immediately prior to use)

6) Sample was added to a Ribolyser tube (Hybaid) and vortexed for 2 s to mix.

7) The sample was processed in Ribolyser for 20 s at 4.0 g.
8) Samples were heated at 100°C for 5 min.
9) A hole was made with a syringe needle on the bottom of Ribolyser tube, it was fitted into a 2 ml Eppendorf tube and placed into a 50 ml Falcon tube which was centrifuged in a benchtop centrifuge at 3000 rpm for 1 min.
10) The flow-through was centrifuged in the Eppendorf tube at top speed in a microcentrifuge for 5 min to pellet debris prior to use.

6.2.1.25 Western Blotting

Typically the amount of protein loaded for Western analysis was 10 μg. Either the following method or a Western Breeze kit (Invitrogen) was used. The Western Breeze kit was performed as described in the manufacturers instructions. The other method is as follows:

1) A protein gel was run (see section 6.2.1.21), and the separating gel was placed in chilled transfer buffer (see Table 6.1) on a shaking platform for 15 min.
2) A 48 mm x 82 mm piece of PVDF membrane (Bio-Rad) was placed in methanol for a few seconds, then in dH_20 for a few minutes and then into chilled transfer buffer on a shaking platform for 10 min.
3) Two sheets of Whatman 3MM paper (70 mm x 100 mm) and the blotting pads were also immersed in chilled transfer buffer.
4) The apparatus was assembled as described in the manufacturers instructions.
5) The blot was run with stirring at 100 V for 90min.
6) The apparatus was dismantled and the membrane was placed in PBST (see Table 6.1) with 5% Marvel (blocking solution), and incubated for 1 hr.
7) The membrane was rinsed twice with PBST, washed for 15 min once and twice for 5 min with PBST.
8) The membrane was then incubated with primary antibody for 1 hr. Primary antibody was typically used at a 1:5000 dilution in PBST.
9) Step 7 was repeated.

10) The membrane was incubated with secondary antibody for 1 hr. Secondary antibody was typically anti-mouse or anti-rabbit HRP conjugated antibody and used at a 1:5000 dilution in PBST.
11) Step 7 was repeated.
12) Detection was performed with ECL detection solution obtained from the ECL™ Western Blotting Detction Reagents Kit (Amersham Pharmacia Biotech) and the manufacturers instructions were followed.
13) The blot was exposed to Hyperfilm™ ECL™ (Amersham Pharmacia) film and developed with a Konica SRX-101A developer.

The above method is slightly different for the analysis of *S. cerevisiae* protein extracts. For *S. cerevisiae* the incubation time in blocking solution is only 30 min, there is no washing after this incubation and the primary antibody is incubated in blocking solution (PBST with 5% Marvel) overnight. The rest of the protocol is as above.

6.2.2 Methods Specific to This Study

6.2.2.1 Pentapeptide Insertion Mutagenesis

The pentapeptide insertion mutagenesis method was used extensively in this study. It is a method of creating random mutations in a gene of interest with the end result being the in-frame insertion of five amino acids. The only limitation of this method is that the gene of interest cannot have a *Kpn*I restriction enzyme site. The method is described in (Hallet *et al.,* 1997; Cao *et al.,* 1997; Hayes *et al.,* 1997).

The method is a transposon based mutagenesis system. The gene of interest was cloned into the plasmid pBR322 and was transformed (see section 6.2.1.1) into the *E. coli* strain FH1046 which also contains the transposon Tn4430Ω5 in the pHT385 plasmid. Several independent transformants were mated (see section 6.2.1.4) to the *E. coli* strain DS941. The mated cells were scraped off the plate and resuspended into 300 µl of LB. 100 µl of the cell suspension was plated onto LB+Strep+Amp+Kan, selecting for donor strain (DS941), the plasmid with the gene of interest, and for the

transposon Tn4430Ω5, respectively. The colonies that were able to grow under selection were picked, grown in liquid culture and the plasmid DNA was extracted (section 6.2.1.5). The extracted DNA was checked to see if the insert was within the gene of interest or the rest of the plasmid. For this the plasmid was digested with *Bam*HI, which will remove the insert from pBR322, and checked for a size difference compared to w.t. *cdc1*. After the screening a *Kpn*I digest was performed (see section 6.2.1.17) to delete the bulk of the transposon, then the plasmid was self-ligated (see section 6.2.1.19) and transformed back into *E. coli* JM109 (see Table 6.6). The resulting plasmid contains the random insertion of five amino acids.

The resulting mutated pBR322-Cdc1 plasmids were digested (see section 6.2.1.17) with *Not*I and *Bam*HI, it was run on a gel and the 1.5 kb insert band was gel extracted (see section 6.2.1.14). This was ligated (see section 6.2.1.19) into pREP3xH_6BN that was digested with *Not*I and *Bam*HI and SAP treated (see section 6.2.1.18). The resulting plasmid was checked by restriction digest with *Bam*HI and *Not*I.

6.2.2.2 *cdc1Δ* Rescue Screen

This protocol was followed to investigate if the *cdc1* mutants were able to rescue *cdc1Δ*. Plasmids carrying mutated *cdc1* (pREP3xH_6BN, see Table 6.4) were individually transformed by electroporation (see section 6.2.1.6) into *cdc1Δ* (a diploid strain where one *cdc1* allele had been replaced with the *ura4+* marker and is leu-, ura+ and ade+, when diploid; see Table 6.7) and plated onto media lacking leucine to select for the plasmids (EMM; see section 6.1.6.2). Four colonies were used to make four patches of the transformants on another EMM plate. This was then replica plated onto ME media (sporulation media; see section 6.1.6.2) and grown for 2-4 days. The spores were helicased (see section 6.2.1.8) and were plated onto two types of media: one plate containing adenine (as the cell will now be haploid and therefore ade-) and uracil (EMM+A+U); the other plate containing adenine and no uracil (EMM+A). Ability to rescue was concluded from the difference in the number of colonies between EMM+A plates and EMM+A+U, see chapter 4 for more details. A sample of colonies growing on the media lacking uracil was checked to ensure

they were haploids. This was carried out in two ways: 1) by growing on media without ade; if they grow on ade- media they are ade+ and hence diploid and 2) by growing in ME and checking for spores; if able to sporulate they are diploid. The above screen was also done simultaneously with media containing thiamine (to repress the nmt promoter) in all media excepting ME.

6.2.2.3 Two-hybrid screen

This method was used to test interactions between prey proteins and the FRYL library (Fromont-Racine *et al.,* 1997) via mating. It can be applied to other libraries.

1) L40 cells (see Table 6.8) containing the pBTM116-Pol31 plasmid (bait cells) were inoculated into 200 ml of the appropriate media and were grown at 30°C with shaking to an OD_{600} of 0.8-1.0.
2) A 2 ml aliquot of FRYL library was thawed on ice. This was inoculated into 20 ml of YPDA+Tet, and incubated at 30°C for 15 min, with gentle shaking (~120 rpm).
3) An equivalent of 80 OD_{600} units (~ 8 x 10^8 cells) of bait cells were mixed with the library containing cells.
4) The cells were concentrated on twelve Millipore filters (ϕ45 mm filters, 0.22 μm). Before use, the filters were prewet with 5 ml of fresh YPDA + Tet. After use each filter was washed with 5 ml of fresh YPDA + Tet.
5) Filters were placed 5 hrs on solid YPDA +Tet and incubated for 5 hrs at 30°C. Filters were cell side up, i.e. not in contact with the media, and it was ensured that there were no air bubbles between the media and the filter.
6) Cells were collected by washing from the filter with YMM –LWH into a total volume of 24 ml.
7) The collected cells were mixed thoroughly, and 50 μl removed for control plates (see below).
8) The mated cells were spread onto YMM-LWH+Ade +3-AT medium, 250 μl per plate, and they were incubated at 30°C for 3 days.

The concentration of 3-AT that was used for the full two-hybrid screen was determined by the mini-screen (see chapter 2). In this case it was found to be 5mM.

For control plates:

1) The 50 µl of cells obtained in step 7 above were diluted 1:1000 by serial dilutions in YMM.
2) 50 µl was plated onto each of these media;

YMM –L+Ade

YMM –W+Ade

YMM –LW+Ade

3) Plates were incubated at 30°C for 2 days.

The following formulae were used to determine the number of diploids screened and the mating efficiency.

$$\% \text{ Mating Efficiency} = \frac{\text{Number of Colonies on –LW}}{\text{Number of Colonies on –L}} \times 100$$

Diploids Screened =

Number of Colonies on YMM -LW x Dilution factor x Volume of culture.

6.2.2.4 Direct Mating of 2-hybrid Interactors

This direct mating method was used to double check interactions found by the two-hybrid method. The direct matings were performed between L40 cells transformed with the pBTM116 plasmid expressing the LexABD-tagged protein to be tested and a strain of opposite mating type (Y187) transformed with the pACT plasmid expressing the Gal4AD-tagged protein to be tested.

1) The L40 strain containing the pBTM116 plasmids were streaked vertically on a YPDA plate. When more that one construct was to be tested multiple streaks were made on the same plate. See Figure 6.1.

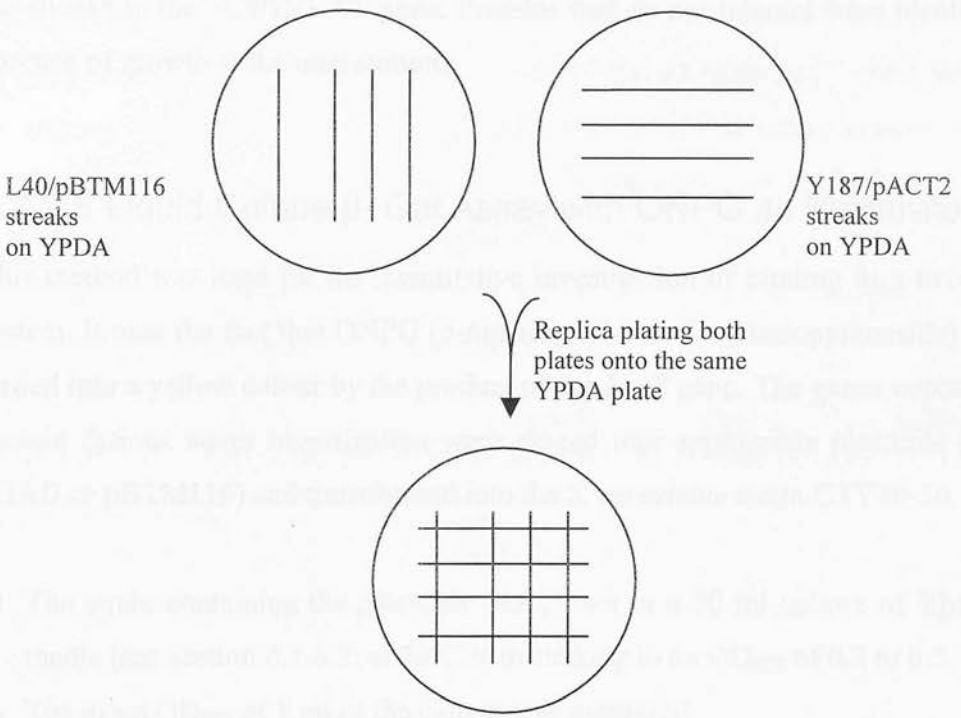

Figure 6.1. Diagrammatic representation of the Direct Mating method of testing two-hybrid interactors.

2) On another YPDA plate, the Y187 containing pACT constructs, were streaked horizontally. See Figure 6.1.
3) Both plates were replica plated onto the same velvet and then onto one YPDA plate. The end result was a single plate with both set of streaks intersecting at some points with each other. See Figure 6.1.
4) This final plate was incubated overnight at 32°C, to allow mating between the strains.
5) This plate was replica plated onto –LW media and –LWH/3-AT media.

The –LW plate is the mating control plate where successful mating was shown by the presence of growth at the intersection of the streaks. The –LWH/3-AT plate additionally selects for activation of the *HIS* reporter gene indicating a positive protein interaction. 3-AT concentration was the same as for a full two-hybrid screen (see section 6.2.2.3). Positive interactions were identified by growth at intersection of the streaks in the –LWH/3-AT plate. Proteins that do not interact were identified by absence of growth at the intersections.

6.2.2.5 Liquid Culture β- Gal Assay with ONPG as Substrate

This method was used for the quantitative investigation of binding in a two-hybrid system. It uses the fact that ONPG (*o*-nitrophenyl-beta-D-galactopyranoside) will be turned into a yellow colour by the product of the *LacZ* gene. The genes encoding the protein fusions under investigation were cloned into appropriate plasmids (pACT, pGAD or pBTM116) and transformed into the *S. cerevisiae* strain CTY10-5d.

1) The strain containing the plasmids was grown in a 50 ml culture of YMM-LW media (see section 6.1.6.2) at 30°C with shaking to an OD_{600} of 0.2 to 0.5.
2) The exact OD_{600} of 1 ml of the culture was measured.
3) The cultures were centrifuged at top speed in a benchtop centrifuge for 5 min and the supernatant was discarded.
4) The pellet was resuspended in 1.5 ml of Z- buffer with β-mercaptoethanol added.

Z- Buffer:

$Na_2HPO4{\cdot}7H_20$	16.1 g/L
$NaH_2PO4{\cdot}\ H_20$	5.5 g/L
KCl	0.75 g/L
$MgSO4{\cdot}7\ H_20$	0.246 g/L

pH was adjusted to 7.0 and autoclaved.

27 µl of β- mercaptoethanol per 10 ml of Z- buffer was added prior to use.

5) The sample was split into three 500 µl aliquots, so that every sample was assayed in triplicate.
6) 10 µl of chloroform was added to each 500 µl sample and it was vortexed for 20s. Care was taken to try to maintain standard vortexing conditions.
7) 100 µl of ONPG (4 mg/ml) was added to each sample, immediately incubated at 30°C. This was taken as time zero.
8) All the reactions were stopped after one of the samples had turned yellow by adding 250 µl of 1 M Na_2CO_3 and incubating on ice. The time elapsed was noted. In the case of proteins that are not interacting samples were stopped after 30 min. Typically the samples were incubated at 30°C for 3-30 min.
9) The samples were centrifuged at top speed in a microfuge for 10 min.
10) The OD_{420} of the supernatant was measured.

The results were expressed as β- Gal units. These units were obtained by using the following formula:

β- Gal units= $1000 \times OD_{420} / (t \times V \times OD_{600})$

OD_{420}= OD_{420} of the supernatant
t= time in minutes
V= volume of cells (typically 16.67)
OD_{600}= OD_{600} of 1 ml of the initial culture (as determined in step 2)

The results were routinely plotted in a bar chart using SigmaPlot for Windows Version 4.00. In some cases such a large number of samples had to be assayed it was not possible to do all of them at the same time. In order to compare samples from different assays a positive control was performed with each set of samples. The mean of the three positive control values was taken to be 100% and the mean of the three sample values was expressed as a percentage of the positive control.

6.2.2.6 PCR overlap extension mutagenesis

This method was described in Newton and Graham, (Newton and Graham, 1997). For this method four primers were needed. Two of them were normal amplification primers of about 15 nucleotides in length, corresponding to primers 1 and 4 in Figure 6.2, below. The other two primers contained the desired mutation and 14 to 15 bases flanking the mutation.

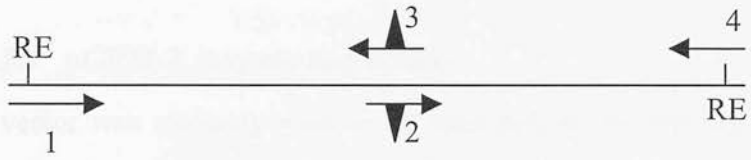

Figure 6.2. Primers used in the PCR extension mutagenesis system. RE is the position of the restriction enzyme sites. 2 and 3 are the mutagenic primers.

PCR reaction were carried out with either Deep Vent or *Pfu* polymerase. Two PCR reactions were performed (see section 6.2.1.15): one with primers 1 and 3 and another one with primers 2 and 4. The reactions were run on a gel and the products were excised. They were placed into an Eppendorf tube, covered with dH_20 and incubated at 37°C for 15 min. The DNA will diffuse out of the gel slices into the dH_20. The DNA contain homologous regions so the two DNA fragments will anneal to each other. Using this aqueous DNA solution as the template, a second round of PCR was performed using primers 1 and 4, resulting in the entire region containing the mutation being amplified. Restriction digests (section 6.2.1.17) were now performed with the enzymes to the sites flanking the mutated region, and the wild-type region was replaced by this mutated one, by further restriction digests and ligation (section 6.2.1.19).

6.2.2.7 Plasmid Multiple Cloning Site Modification

This protocol was used to modify the polylinker of a plasmid, usually to modify the reading frame or a restriction enzyme site. The plasmid to be modified was digested

(see section 6.2.1.17) with an appropriate restriction enzyme. Oligonucleotides were designed so that when annealed (as described in section 6.2.1.20) the desired multiple cloning site was created. The design of the primers was also such that the annealed primers had overhangs corresponding to those of the digested plasmid. The annealed primers were ligated into the digested plasmid (see section 6.2.1.19).

6.2.2.8 Cloning Strategies

6.2.2.8.1 pGEM-T easy cloning vector

This vector was routinely used as an intermediate for cloning, specially when the fragment to be cloned was a PCR product. pGEM-T was obtained from Promega and the manufacturers instructions were followed.

6.2.2.8.2 Cloning *cdc1* t.s. mutants

Sequences encoding *cdc1* from the three t.s. strains *cdc1-A24*, *cdc1-64* and *cdc1-223* were amplified in two halves by PCR (see section 6.2.1.15). The 5' region was amplified with the primers CDC15'U and CDC15'L. The 3' region was amplified with the primers CDC13'U and CDC13'L. The 5' PCR fragments were digested with *Xmn*I and *Hind*III. This was ligated into pTZ19R digested with *Sma*I and *Hind*III. (*Xmn*I and *Sma*I both produce blunt ends). The 3' PCR fragments were digested with *Hind*III and *Sal*I and was ligated into pTZ19R digested with *Hind*III and *Sal*I. See Table 6.2 for the sequence of the primers.

6.2.2.8.3 Cloning Pol3CT from *S. pombe*

Sequences encoding Pol3CT were cloned by amplifying pREP81X-Pol3 with the Pol3CT1 and nmt3' primers (see Table 6.2). The PCR fragment was ligated into the easy cloning vector pGEM-T (Promega; see section 6.2.2.8.1). The resulting plasmid was digested with *Bam*HI and the DNA fragment was ligated into *Bam*HI digested pGEX6P-1B. The final plasmid was checked by amplifying with the GEX3' and the

Pol3CT primers and subsequent sequencing with the Pol3CT primer. The insert from this plasmid was subcloned into pACT2 by Dr. S. MacNeill.

6.2.2.8.4 Subcloning ZnF2 into pACT2

The plasmid pGEX6P1B-ZnF2 was digested with *Bam*HI. The digest was run on a gel, the insert band was gel extracted (see section 6.2.1.14) and ligated (see section 6.2.1.19) into *Bam*HI digested, SAP treated (see section 6.2.1.18) pACT2. The resulting plasmid was checked by PCR with pACT3' and ZnF2F primers (see Table 6.2) and by restriction digest with *Bam*HI.

6.2.2.8.5 Cloning of ScPol3 and ScPol3ΔZnF2

Due to the large size of Pol3 it was decided to clone sequences encoding it in two halves by PCR (see section 6.2.1.15). For ScPol3 the primers used were ScPol3 5' and ScPol3B for the amplification of the 5' portion of the gene; primers ScPol3A and ScPol3 3' were used for the amplification of the 3' end of the gene; primers ScPol3A and ScPol3ΔZnF2 were used for the amplification of the 3' end of the gene with ZnF2 deleted. Table 6.2 shows the sequence of the primers.

The two individual halves of Pol3 were ligated (see section 6.2.1.19) into pGEM-T (see section 6.2.2.8.1) and were checked by sequencing (see section 6.2.1.16) with the oligos that were used in the amplification and with ScPol3SEQ1, ScPol3SEQ2, ScPol3SEQ3, ScPol3SEQ4 (see Table 6.2). The two halves of Pol3 were removed by restriction digest (see section 6.2.1.17; the 5' half with *Xma*I and *Bsm*I and the 3' half with *Bsm*I and *Eco*RI) and cloned by ligating both fragments simultaneously into *Xma*I and *Eco*RI digested (see section 6.2.1.17) and SAP treated (see section 6.2.1.18) pACT2.

The result is the full length wild type *S. cerevisiae* Pol3 and *S. cerevisiae* Pol3ΔZnF2 in which the last 53 amino acids have been removed, the C- terminal Pol3 amino acids are: Asp, Val, Arg, and Asp (the last amino acid is amino acid number 1044). However, due to not placing a stop codon in the primer this results in the addition of

the following amino acids: Glu, Phe, Glu, Leu, Glu, Arg, Ser, Met, Asn, Arg, Arg, Tyr, after which a stop codon was present in frame, as checked by sequencing (see section 6.2.1.16).

6.2.2.8.6 Cloning DomIII

Sequences encoding the desired region (DomIII and some flanking amino acids: from amino acid 108-171) were amplified by PCR (see section 6.2.1.15) using the DomIII F and DomIII B primers (see Table 6.2). The PCR fragment was digested with *Bam*HI (see section 6.2.1.17), ligated into pGEX6P-1B and checked by sequencing (see section 6.2.1.16). Due to not placing a stop codon in the backward primer this resulted in the addition of the following amino acids: Gly, Ser, Pro, Ala, Ala, and Ser, after which a stop codon is located. It is not thought that the presence of these amino acids will alter the properties of DomIII as these amino acids appear within the DomIII region. DomIII was then subcloned into pBTM116 by restriction digest with *Bam*HI and ligation into pBTM116.

6.2.2.8.7 Creation and Cloning of Sdp5

This mutation was created by using the PCR overlap extension mutagenesis system (see section 6.2.2.6). The non mutagenic primers (oligosnucleotides 1 and 4 in Figure 6.2) were pBTM1165' and BTM3' (see Table 6.2) and the mutagenic primer pair were Sdp5F and Sdp5B (see Table 6.3). The template for this reaction was pBTM116-Pol31. The PCR products were digested with *Eco*RI. pBTM116-Pol31 was digested with *Eco*RI (see section 6.2.1.17), was SAP treated (see section 6.2.1.18) and the 6kb vector fragment was purified by gel extraction (see section 6.2.1.14). The PCR product was ligated into this plasmid. The resulting plasmid was checked by restriction digest with *Bam*HI and sequencing with pBTM1165', BTM3', Pol31SEQ1 and Pol31SEQ2 oligos (see Table 6.2).

6.2.2.8.8 Cloning Cdm1

Sequences encoding Cdm1 were amplified by PCR with the following primers: Cdm1MOD5 and nmt3' (see Table 6.2) with pREP3xH_6Cdm1 as template. It was cloned into pGEM-T (see section 6.2.2.8.1). This vector was digested (see section 6.2.1.17) with *Bam*HI and ligated into *Bam*HI digested SAP treated (see section 6.2.1.18) pAA. The resulting plasmid was sequenced with nmt5' and Cdm1B2 primers (see Table 6.2).

6.2.2.8.9 Site Directed Mutagenesis of Cdc1

The site directed mutants of *cdc1* were performed with a Mutagene Phagemid *in vitro* mutagenesis kit (BioRad), following the manufacturers instructions. The mutagenic oligos used for each of the mutations are detailed in Table 6.9 (see Table 6.3 for their sequence):

Table 6.9. Mutagenic oligonucleotides used in the Cdc1 site directed mutagenesis

Mutation Name	Mutagenic oligonucleotide
A1	AANO1
A2	AANO2
A3	AANO3
A4	AANO4
A5	AANO5
A6	AANO6
A7	AANO7
A8	AANO8
A9	AANO1/2
A10	AANO5/6

The resulting mutated pBR322-Cdc1 plasmids were digested (see section 6.2.1.17) with *Not*I and with *Bam*HI, it was run on a gel and the 1.5kb insert band was gel extracted (see section 6.2.1.14). This was ligated (see section 6.2.1.19) into pREP3xH_6BN that was digested with *Not*I and *Bam*HI and SAP treated (see section 6.2.1.18). The resulting plasmid was checked by restrcition digest with *Bam*HI and *Not*I.

6.2.2.8.10 Subcloning Cdc1 mutants into pBTM

To subclone the pREP3xH_6BN-Cdc1 mutants into pBTM116, first the multiple cloning site of pBTM116 was modified. The modification was done with pBTMMOD1 and pBTMMOD2 primers (see Table 6.3) and the method described in section 6.2.2.7. This modified vector was digested (see section 6.2.1.17) with *Not*I and *Bam*HI and was SAP treated (see section 6.2.1.18). The pREP3xH_6BN-Cdc1 mutants were digested (see section 6.2.1.17) with *Not*I and *Bam*HI, it was run on a gel and the 1.5kb insert band was gel extracted (see section 6.2.1.14). This purified DNA was ligated into the digested and SAP treated modified pBTM116 vector. The resulting plasmids were checked by restriction digests with *Bam*HI and *Not*I.

6.2.2.8.11 Mutagenesis of ZnF2

The mutagenesis of both *S. cerevisiae* and *S. pombe* ZnF2 was performed using the PCR overlap extension mutagenesis system (see section 6.2.2.6). The non mutagenic primers (oligonucleotides 1 and 4 in Figure 6.2) were pACT5' and pACT3'. The mutagenic primers for each of the mutations were the following ("F" is the forward primer and "B" is the backward primer):

Table 6.10. Mutagenic oligonucleotide pairs used in the mutagenesis of both *S. pombe* and *S. cerevisiae* ZnF2.

Mutation Name	Mutagenic primer pair	Mutation Name	Mutagenic primer pair
SpCys1A	SpCys1AF SpCys1AB	R1051A	ARG1F ARG1B
SpCys2A	SpCys2AF SpCys2AB	W1053A	TRPF TRPB
SpCys3A	SpCys3AF SpCys3AB	C1056A	CYS1F CYS1B
SpCys4A	SpCys4AF SpCys4AB	C1056S	CYS1F CYS1B
SpCys4S	SpCys4SF SpCys4SB	C1059S	CYS2F CYS2B
		H1064A	HISF HISB
		E1066A	GLUF GLUB
		C1069A	CYS3F

			CYS3B
		C1069S	CYS3F CYS3B
		C1074A	CYS4F CYS4B
		C1074S	CYS4F CYS4B
		F1077A-Y1078A	PHETYRF PHETYRB
		R1080A	ARG2F ARG2B

The PCR fragments were digested with *Bam*HI (see section 6.2.1.17) and ligated (see section 6.2.1.19) into *Bam*HI digested, SAP treated (see section 6.2.1.18) pACT2. The fragments were ligated by PCR with a mutagenic backward primer and pACT5'. They were also checked by sequencing with pACT5'.

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8 Appendix A. Raw data

8.1 Raw Data for Chapter 2

Raw data for liquid culture β- Gal assay performed on the two hybrid interactors.				
Two-hybrid protein fusions	β- Gal Units with LexABD-Pol31	Mean of β- Gal Units with LexABD-Pol31	β- Gal Units with LexABD	Mean of β- Gal Units with LexABD
Gal4AD-9	99.100 92.880 83.780	91.92	1.070 1.160 1.050	1.09
Gal4AD-20	30.820 30.920 31.030	30.92	15.690 16.150 15.800	15.88
Gal4AD	0.259 0.243 0.248	0.25	0.459 0.459 0.416	0.445

Table 8.1. Raw data for liquid culture β- Gal assay performed on the two-hybrid interactors. Gal4AD-9is the smallest clone and Gal4AD-20 is the biggest, corresponding to both C- terminal zinc fingers. See Figure 2.3.

Binding of ZnF2 mutations to LexABD-Pol31 and LexABD-Sdp5.			
ZnF2 mutation	% of wild type with LexABD-Pol31	% of wild type with LexABD-Sdp5	Increase in LexABD-Sdp5
W.T.	100	100	0
E1046	0.165	3.190	3.025
E1047	12.180	59.050	46.87
K1048	51.250	70.970	19.72
R1051	19.850	32.480	12.63
L1052	39.000	82.800	43.8
W1053	37.550	42.860	5.31
T1054	36.630	59.770	23.14
Q1055	56.450	77.780	21.33
C1056A	1.250	7.310	6.06
C1056S	0.935	12.550	11.615
Q1057	13.710	44.500	30.8
R1058	8.460	21.210	12.75
C1059S	2.140	24.880	22.74
N1062	47.320	80.630	33.31
H1064	19.570	62.860	43.29
E1066	108.910	94.940	-13.97
C1069A	0.365	1.060	0.692
C1069S	0.373	1.370	0.997
K1072	90.780	91.790	1.01
C1074A	0.733	1.110	0.377
C1074S	0.242	1.180	0.938
F1077Y1078	34.860	30.100	-4.76
R1080	113.120	114.070	0.95
1085 Stop	0.162	0.265	0.103

Table 8.2. Binding of ZnF2 mutations to LexABD-Pol31 and LexABD-Sdp5. Values are in percentages comparing the ZnF2 mutations with the wild type, where wild type binding is 100%. See Figures 2.6 and 2.7

Raw data for liquid culture β- Gal assay done with LexABD-Pol3 and LexABD-Pol3ΔZnF2.				
Pol3 protein fusions	β- Gal Units with LexABD-Pol31	Mean of β- Gal Units with LexABD-Pol31	β- Gal Units with LexABD	Mean of β- Gal Units with LexABD
Gal4AD-Pol3	15.00 15.62 15.27	15.30	0.193 0.225 0.418	0.279
Gal4AD-Pol3ΔZnF2	0.244 0.259 0.320	0.274	0.297 0.149 0.204	0.217
Gal4AD	0.302 0.264 0.283	0.283	0.492 0.484 0.463	0.480

Table 8.3. Raw data for liquid culture β- Gal assay done with Gal4AD-Pol3 and Gal4AD-Pol3ΔZnF2. See Figure 2.8.

8.2 Raw Data for Chapter 3

Liquid culture β- Gal assay to investigate the binding of Gal4AD-Pol3 to LexABD-Cdc1.		
	β- Gal Units	Mean of β- Gal Units
Gal4AD-Pol3 LexABD-Cdc1 Myc	0.095 0.076 0.061	0.077
Gal4AD-Pol3 LexABD-Cdc1 Myc-Cdc27	3.340 3.310 3.230	3.29
Gal4AD-Pol3 LexABD Myc-Cdc27	0.747 0.764 0.893	0.801
Gal4AD LexABD-Cdc1 Myc-Cdc27	0.073 0.049 0.116	0.079
Gal4AD LexABD Myc	0.091 0.107 0.086	0.095

Table 8.4. Liquid culture β- Gal assay to investigate the binding of Gal4AD-Pol3 to LexABD-Cdc1. Myc is expressed from the empty vector pAA. See Figure 3.1.

Liquid culture β- Gal assay to investigate the binding of Gal4AD-Pol3CT to LexABD-Cdc1.		
	β- Gal Units	Mean of β- Gal Units
Gal4AD-Pol3CT LexABD-Cdc1 Myc	0.113 0.143 0.166	0.141
Gal4AD-Pol3CT LexABD-Cdc1 Myc-Cdc27	115.34 104.56 109.95	109.95
Gal4AD LexABD-Cdc1 Myc-Cdc27	0.000 0.066 0.022	0.029
Gal4AD-Pol3CT LexABD Myc-Cdc27	2.025 2.018 2.039	2.027
Gal4AD LexABD Myc	0.052 0.061 0.061	0.058
Gal4AD-Pol3CT LexABD-Cdc1Δ453 Myc-Cdc27	0.097 0.106 0.115	0.106

Table 8.5. Liquid culture β- Gal assay to investigate the binding of Gal4AD-Pol3CT to LexABD-Cdc1. See Figure 3.4.

Liquid culture β- Gal assay to investigate the binding of Gal4AD-ZnF2 to LexABD-Cdc1		
	β- Gal Units	Mean of β- Gal Units
Gal4AD-ZnF2 LexABD-Cdc1 Myc-Cdc27	0.687 0.363 0.592	0.547
Gal4AD LexABD-Cdc1 Myc-Cdc27	0.312 0.378 0.334	0.341
Gal4AD-ZnF2 LexABD Myc-Cdc27	0.524 0.580 0.716	0.607
Gal4AD-ZnF2 LexABD-Cdc1 Myc	0.396 0.318 0.387	0.367
Gal4AD LexABD Myc	0.737 0.343 0.506	0.529
Gal4AD-Pol3CT LexABD-Cdc1 Myc-Cdc27	71.76 73.79 59.03	68.19

Table 8.6. Liquid culture β- Gal assay to investigate the binding of Gal4AD-ZnF2 to LexABD-Cdc1. See Figure 3.5.

Liquid culture β- gal assay to investigate possible stabilising effect of myc-tagged Cdm1.		
	β- Gal Units	Mean of β- Gal Units
Gal4AD-Pol3CT LexABD-Cdc1 Myc-Cdm1	0.135 0.199 0.163	0.166
Gal4AD-Pol3CT LexABD-Cdc1 Myc-Cdc27	72.34 66.67 68.09	69.03
Gal4AD-Pol3CT LexABD-Cdc1 Myc	0.172 0.165 0.330	0.222
Gal4AD-Pol3CT LexABD Myc-Cdm1	0.605 0.650 0.654	0.636
Gal4AD LexABD-Cdc1 Myc-Cdm1	0.154 0.173 0.125	0.151
Gal4AD LexABD Myc	0.170 0.158 0.243	0.190

Table 8.7. Liquid culture β- gal assay to investigate possible stabilising effect of myc-tagged Cdm1. See Figure 3.8

Liquid culture β- Gal assay to investigate the binding of Gal4AD-ZnF2 mutants to LexABD-Cdc1.		
	β- Gal Units	Mean of β- Gal Units
Gal4AD-Pol3CT LexABD-Cdc1 Myc-Cdc27	65.19 64.18	64.69
Gal4AD-SpCys1A LexABD-Cdc1 Myc-Cdc27	0.448 0.900	0.674
Gal4AD-SpCys2A LexABD-Cdc1 Myc-Cdc27	0.390 0.570	0.480
Gal4AD-SpCys3A LexABD-Cdc1 Myc-Cdc27	1.050 0.689	0.870
Gal4AD-SpCys4A LexABD-Cdc1 Myc-Cdc27	0.423 0.599	0.511
Gal4AD-SpCys4S LexABD-Cdc1 Myc-Cdc27	0.805 0.776	0.791
Gal4AD LexABD-Cdc1 Myc-Cdc27	0.866 0.705	0.786
Gal4AD-Pol3CT LexABD Myc-Cdc27	0.894 3.920	2.41
Gal4AD-Pol3CT LexABD-Cdc1 Myc	0.827 1.030	0.929
Gal4AD LexABD Myc	0.708 1.330	1.02

Table 8.8. Liquid culture β- Gal assay to investigate the binding of Gal4AD-ZnF2 mutants to LexABD-Cdc1. See Figure 3.9.

8.3 Raw Data for Chapter 4

Ability of the Cdc1 mutants to rescue Cdc1Δ

Mutant	Colonies on EMM+A+U	Colonies on EMM+A	Rescue with promoter derepressed	Colonies on EMM+A+ U+T	Colonies on EMM+A+T	Rescue with promoter repressed
J1	>>1000	100	No	>>1000	<100	No
J2	>>1000	>>1000	Yes	>>1000	>>1000	Yes
J3	>>1000	>>1000	Yes	>>1000	<100	No
J4	>>1000	>>1000	Yes	>>1000	<100	No
J5	>>1000	>>1000	Yes	>>1000	<100	No
J6	>>1000	>>1000	Yes	>>1000	<100	No
J7	>>1000	2	No	>>1000	<100	No
J8	>>1000	>>1000	Yes	>>1000	>1000	Yes
J9	>>1000	>>1000	Yes	>>1000	>>1000	Yes
J10	>>1000	>>1000	Yes	>>1000	>100	No
J11	>>1000	>>1000	Yes	>>1000	<100	No
J12	>>1000	>>1000	Yes	>>1000	>1000	Yes
J13	>>1000	>>1000	Yes	>>1000	100	No
J15	>>1000	>>1000	Yes	>>1000	>1000	Yes
J16	>>1000	<100	No	>>1000	>100	No
J17	>>1000	>>1000	Yes	>>1000	>100	No
J18	>>1000	>>1000	Yes	>>1000	>1000	Yes
J19	>>1000	>>1000	Yes	>>1000	>1000	Yes
J20	>>1000	>>1000	Yes	>>1000	>1000	Yes
J21	>>1000	>>1000	Yes	>>1000	>100	No
J22	>>1000	>>1000	Yes	>>1000	>1000	Yes

Ability of the Cdc1 mutants to rescue Cdc1Δ (contd.)						
Mutant	Colonies on EMM+A+U	Colonies on EMM+A	Rescue with promoter derepressed	Colonies on EMM+A+U+T	Colonies on EMM+A+T	Rescue with promoter repressed
E1	>1000	>1000	Yes	>>1000	>1000	Yes
E2	>1000	11	No	>>1000	37	No
E3	>1000	>1000	Yes	>>1000	<100	No
E4	>1000	>1000	Yes	>>1000	<100	Yes
E5	>1000	>1000	Yes	>>1000	>1000	Yes
E6	>1000	>1000	Yes	>>1000	10	No
E7	>1000	>1000	Yes	>>1000	12	No
E8	>1000	>1000	Yes	>>1000	38	No
E9	>1000	>1000	Yes	>>1000	<100	No
E10	>1000	>1000	Yes	>>1000	>1000	Yes
A1	>>1000	>>1000	Yes	>>1000	>>1000	Yes
A2	>>1000	>>100	Yes	>>1000	31*	Yes
A3	>>1000	1	No	>>1000	34	No
A4	>>1000	>>1000	Yes	>>1000	94*	Yes
A5	>>1000	>>1000	Yes	>>1000	>>100	Yes
A6	>1000	>1000	Yes	>>1000	30	No
A7	>>1000	>>1000	Yes	>>1000	>100*	Yes
A8	>>1000	>>1000	Yes	>>1000	>1000	Yes
A9	>>1000	85	No	>>1000	49	No
A10	>>1000	0	No	>>1000	37	No

Table 8.9. Ability of helicased spores to grow on the mentioned media. ">>", much more than. * All checked haploid. See chapter 4 for more details.

Ability of the Cdc1 "J" mutants to rescue *cdc1-P13*						
	28°C EMM	28°C EMM+ T	36.5°C EMM	36.5°C EMM+ T	Rescue with promoter derepressed	Rescue with promoter repressed
Plasmid	Growth	Growth	Growth	Growth		
Prep3xH$_6$BN 3xcdc1	+++	+++	-	-	No	No
J1	+++	+++	+++	+++	Yes	Yes
J2	+++	+++	-	-	No	No
J3	+++	+++	+++	+	Yes	Yes
J4	+++	+++	+++	+	Yes	Yes
J5	+++	+++	+++	++	Yes	Yes
J6	+++	+++	+++	+	Yes	Yes
J7	-	+++	-	-	No	No
J8	+++	+++	+++	+	No	Yes
J9	+++	+++	+++	++	Yes	Yes
J10	+++	+++	++	+++	Yes	Yes
J11	+++	+++	++	+	Yes	Yes
J12	+++	+++	+++	+	Yes	Yes
J13	+++	+++	+++	++	Yes	Yes
J15	+++	+++	+++	++	Yes	Yes
J16	+++	+++	+	+++	Yes	Yes
J17	+++	+++	+++	+	Yes	Yes
J18	+++	+++	+++	-	Yes	No
J19	+++	+++	+++	++*	Yes	Yes
J20	+++	+++	+++	++*	Yes	Yes
J21	+++	+++	+++	++*	Yes	Yes
J22	+++	+++	+++	+/++	Yes	Yes
				-	Yes	No

Table 8.10. Ability of the Cdc1 "J" mutants to rescue cdc1-P13, a *cdc1* t.s. strain. +++ indicates good growth, + indicates poor growth and – indicates no growth. * indicates long cells. See section 4.2.3.

Ability of the Cdc1 "A" mutants to rescue *cdc1-P13*						
	28°C EMM	28°C EMM+ T	36.5°C EMM	36.5°C EMM+ T	Rescue with promoter derepressed	Rescue with promoter repressed
Plasmid	Growth	Growth	Growth	Growth		
pREP3xH$_6$BN	+++	+++	-	-	No	No
3xcdc1	+++	+++	+++	+++	Yes	Yes
A1	+++	+++	+++	+++	Yes	Yes
A2	+++	+++	+	+	Yes	Yes
A3	+	+++	-	-	No	No
A4	++	+++	+++	++*	Yes	Yes
A5	++	+++	+++	++*	Yes	Yes
A6	+++	+++	+	+	Yes	Yes
A7	+++	+++	+++	++*	Yes	Yes
A8	+	+++	++	++*	Yes	Yes
A9	++	+++	-	-	No	No
A10	-	+++	-	-	No	No

Table 8.11. Ability of the Cdc1 "A" mutants to rescue cdc1-P13, a *cdc1* t.s. strain. +++ indicates good growth, + indicates poor growth and – indicates no growth. * indicates long cells. See section 4.2.3.

Raw data of the LexABD-Cdc1 mutants and their ability to interact with either Gal4AD-Pol3 or Gal4AD-Cdc27.		
Cdc1 Mutation	% of wild type binding with Gal4AD-Pol3	% of wild type binding with Gal4AD-Cdc27
W.T.	100	100
J12	0.109	58.63
E4	15.62	61.87
J18	46.04	0.408
E2	0.229	0.344
J17	2.29	0.293
E3	53.65	0.615
E1	65.41	1.230
J10	0.069	29.51
J8	0.056	127.90
E5	48.34	88.25
J6	0.161	0.295
E9	23.52	0.271
J5	35.97	0.462
J1	0.054	0.184
J16	0.370	0.301
J9	91.46	102.13
J15	1.23	78.25
J22	24.56	7.18
J21	0.238	0.562
A1	38.98	43.97
A2	0.156	18.67
A9	0.422	58.49
A3	0.179	23.23
A4	0.068	31.13
A5	4.22	36.58
A6	0.162	6.35
A10	0.485	71.40
A7	8.84	0.636
A8	17.03	8.01
J7	0.00	18.62

Table 8.12. Raw data of the LexABD-Cdc1 mutants and their ability to interact with either Gal4AD-Pol3 or Gal4AD-Cdc27. Wild type LexABD-Cdc1 was taken as an interaction of 100%, the mutants were compared to that value. The mutants are ordered according to their position within *cdc1+*, i.e. those in the N- terminal region are at the top of the table etc. See Figure 4.8 and Figure 4.9.

Raw data of the ability of LexABD-DomIII to interact with Gal4AD-Cdc27.		
	β- Gal Units	Mean of β- Gal Units
Gal4AD-Cdc27 LexABD-DomIII	0.612 0.380 0.380	0.457
Gal4AD LexABD-DomIII	0.375 0.323 0.336	0.345
Gal4AD-Cdc27 LexABD	0.538 0.642 0.667	0.616
Gal4AD-Cdc27 LexABD-Cdc1	74.810 74.300 73.790	74.3

Table 8.13. Raw data of the ability of LexABD-DomIII to interact with Gal4AD-Cdc27. Gal4AD is expressed from pGAD the empty vector, LexABD is expressed from the empty vector pBTM116. See Figure 4.10.